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THE MECHANISM OF VITAL STAINING WITH BASIC DYES.

By MORTON McCUTCHEON and BALDWIN LUCKE.

(From the McManes Laboratory of Pathology, School of Medicine, University of Pennsylvania, and the Marine Biological Laboratory, Woods Hole.)

(Received for publication, March 1, 1924.)

That intravitam staining with basic dyes occurs more readily in alkaline than in acid solutions has long been known. Among the explanations which have been offered to account for this fact two deserve especial mention. The first is that originally proposed by Overton (1), accepted by Harvey (2), and supported in certain respects by the experiments of Robertson (3) (who, however, on other grounds arrived at a theory diametrically opposed to that of Overton.) According to Overton's theory, cells are freely permeable to the lipoid-soluble dye base but not to the relatively lipoid-insoluble salts of this base with various acids. In acid solutions the dye is present chiefly in the form of salts which enter cells with difficulty, while in alkaline solutions it exists largely in the form of the readily penetrating free base. The difference in staining in the two cases, according to this theory, is due primarily to factors outside of the cell.

The second view, advocated in recent years among others by Bethe (4), Rohde (5), and Irwin (6, 7), depends on the fact that in relatively alkaline solutions proteins combine readily with basic and not with acid dyes and show the reverse behavior in more acid solutions. (In this connection, see also Loeb, (8).) According to this view the state of the dye in the external solution is of less importance than the condition of the proteins within the cell.

If it were possible to vary experimentally and independently of one another the pH inside and outside of a cell it ought to be possible to decide which of these two theories is more in accord with the facts; since if the first is correct, the degree of staining ought to depend largely on the pH of the external solution, while if the second is correct it ought to depend more on that of the cell itself. More specifically, if two cells, one relatively more alkaline internally than the other,

be exposed to two solutions containing the dye at the same pH, then according to the second theory the more alkaline cell should stain more deeply, while according to the first (in its simplest form) the two cells should stain equally.

It is, as a matter of fact, easy to produce, within the range of reactions tolerated by living cells, almost any desired combination of external and internal pH. It is well known that a weak solution of NH_4OH quickly produces intracellular alkalinity while one of NaOH does not. (Bethe (9), Warburg (10), Harvey (3).) Similarly, carbon dioxide produces intracellular acidity far more readily than a mineral acid such as HCl at the same pH (Jacobs (11)). It has also been shown by Jacobs (11, 12) that where carbon dioxide and ammonia are concerned the internal and the external pH may be entirely independent of one another, a cell becoming acid in an alkaline solution and alkaline in an acid solution.

In the following experiments, the pH of the cells and of the surrounding solutions was regulated in this manner by means of NaOH, NH_4OH, CO_2, and HCl. The dyes employed were neutral red and brilliant cresyl blue, and the living material was: (*a*) starfish eggs, (*b*) *Gonionemus* (entire animals), and (*c*) *Nitella* cells. For the first two types of material the basis of the solutions used was neutralized sea water brought into equilibrium with the air by shaking in a large flask; in the case of *Nitella*, distilled water or boric acid buffer mixture. That the cells were not killed by the treatment they received was shown by the ability of the starfish eggs to undergo cleavage after fertilization, by the movements of locomotion of *Gonionemus*, and by protoplasmic rotation in the case of *Nitella*. Since the results obtained with the three types of cells and with the two dyes agreed in their most essential points it is believed that they represent fairly general conditions.

In general, our results furnish no support for the second theory. If this theory were true, a cell should stain more deeply in a solution of *e.g.* pH 8.0 produced with NH_4OH, than with NaOH, since in the former case the interior of the cell is made more alkaline while in the latter it is not, or at least is not to the same extent. As a matter of fact, in every case, with all three kinds of material, the staining was *less* with NH_4OH than with NaOH as will be apparent from the descriptions of several typical experiments.

Starfish eggs were placed in test-tubes which contained neutralized sea water brought to pH 8.0 (determined colorimetrically) with NaOH in one case, and with NH_4OH in the other. In one series of experiments equal amounts of very dilute brilliant cresyl blue were added, and the eggs stained 2 to 8 minutes. Those in NaOH were then found to be more intensely colored than those in NH_4OH. Determination at pH 9.0 gave the same result.

Using neutral red in alkaline solutions, eggs in NaOH were stained red (the acid color), while those in NH_4OH were yellow (alkaline). To compare the amounts of dye taken up by the two lots of eggs, they were very quickly washed and placed in neutralized sea water, and CO_2 was allowed to bubble through, thus producing an acid reaction inside all of the cells. The eggs stained in NaOH were now more deeply red, and evidently had taken up more dye, than those stained in NH_4OH.

The effect of acids on staining was tested as follows: Neutralized sea water was placed in three beakers. The first solution was acidified with HCl; into the second CO_2 was allowed to bubble. Sodium bicarbonate was added to the third, and then into it CO_2 was passed. The three solutions were brought to pH 6.0. With neutral red, starfish eggs stained more intensely in the CO_2 solution (with or without bicarbonate) than in the HCl solution. In some of the experiments a deeper color was obtained in the bicarbonate-CO_2 mixture than in CO_2 alone. The eggs were stained 2 to 7 minutes. Control beakers showed no change in pH at the end of the experiment.

In another series of experiments *Gonionemus* was stained with brilliant cresyl blue for 15 to 30 minutes at pH 8.0. The solutions were prepared as described above and were placed in tall covered glass cylinders. No appreciable alteration in pH occurred in the same time in control cylinders containing animals but no dye. Under these conditions, in the solution containing NaOH the umbrella, manubrium, tentacles, and especially the velum took up the dye freely, while in the NH_4OH solution the staining was very faint. Owing to the constant movements of the animals it was impossible to determine by microscopic examination the exact point of accumulation of the dye, except to be certain that it was in the interior and not on the surface of the body.

Finally, *Nitella* cells 4 to 5 cm. in length were placed in 50 cc. of 0.01 molar buffer solutions which had been brought to a given pH in the one case with NaOH, in the other with NH_4OH. The dye employed was brilliant cresyl blue. After 30 minutes to 26 hours, the ends of several cells were snipped off, the sap was gently expressed and pooled, drawn up into capillary tubes and compared with various concentrations of the dye contained in tubes of the same diameter. This method allows a quantitative determination of the dye in the sap (Irwin (6, 7)).

In thirteen out of fourteen experiments in which quantitative estimates were made, a higher concentration of the dye (in some cases more than ten times as great) was found in the sap vacuoles of the cells stained in NaOH than in those stained in NH_4OH; in the remaining experiment the concentrations were about equal. In Table I are recorded the results of two typical experiments. It will be noted that while the total amounts of dye taken up in the second case are considerably greater than in the first (due possibly to the fact that the material had been kept for a longer time in the laboratory) the relative concentrations in the NaOH and NH_4OH solutions remain about the same.

TABLE I.

Two typical experiments showing the influence of NaOH and NH_4OH on the pH of the sap of *Nitella*. Cells were stained with brilliant cresyl blue in 0.01 molar solution of boric acid brought to pH 8.0 with either NaOH or NH_4OH. Unstained cells were used for determining the pH of the sap. Time of exposure 1 hr.

Experiment No.	Dye in outside solution.	Dye inside cells in NaOH.	Dye inside cells in NH_4OH.	pH of sap. Cells in NaOH.	pH of sap. Cells in NH_4OH.
	per cent	*per cent*	*per cent*		
1	0.0006	0.003	<0.001	5.2	6.6
2	0.0006	0.020	0.006	5.8	6.3

In two experiments *Nitella* cells were placed in the two solutions, and after a certain time some of them were examined and showed, as usual, more intense staining in NaOH. The remaining cells were then transferred from NaOH into NH_4OH, and conversely, from NH_4OH into a NaOH. Subsequent examination showed that the cells transferred to NH_4OH had lost much of their stain, while those transferred to NaOH were now stained more deeply.

It thus appears that the three kinds of organisms employed stain more intensely with certain basic dyes in NaOH than in NH_4OH solutions of the same pH.

That this difference is not due to some specific effect of NH_3 on the partition of dye between fat and water is made probable by the following experiment. Six solutions were prepared of 0.01 molar boric acid

solution and brought to pH 7.1, 7.8, and 8.8 with NaOH in the one case and with NH_4OH in the other. 10 cc. of each solution were shaken with 1 cc. of oil of sweet almonds, using brilliant cresyl blue as the dye. The intensity of staining of the oil was found to increase with increasing pH, but there was no definite difference between tubes containing NaOH and those containing NH_4OH at the same pH.

The experiments so far described, therefore, furnish no support for the second theory. They might also appear to furnish little support for the first theory. If staining depended merely on the proportions of dye-base and dye-salt in the external solution, then, at the same pH, staining should be equally deep regardless of whether alkalinity were produced with NH_4OH or NaOH, or acidity with CO_2 or HCl. This, as has been mentioned, is not the case. With the external pH the same, staining is more intense with NaOH than with NH_4OH. Evidently in the cases mentioned some other factor or factors must produce or at least modify the observed results.

However, certain other observations favor the first theory. For example, when placed in very weak solutions of NaOH or of HCl, starfish eggs undergo no change in reaction for a considerable time, as may be demonstrated by the use of neutral red as an intracellular indicator. Nevertheless, they stain far more rapidly and deeply in the alkaline than in the acid solution. Similarly, in a series of solutions in which the CO_2 tension is the same, the addition of increasing amounts of $NaHCO_3$ would not, for a time at least, prevent the development of the same degree of intracellular acidity; nevertheless, the presence of bicarbonate in the external solution favors staining as the following experiment shows.

To equal amounts of sea water saturated with CO_2 were added increasing quantities of M/2 $NaHCO_3$; and an equal number of drops of a weak solution of neutral red or brilliant cresyl blue. The pH range obtained was from 5.0 (CO_2 alone) to 6.0. Several specimens of *Gonionemus* were placed in each of the several portions and allowed to remain for 15 to 30 minutes. The intensity of staining was now found to be proportional to the amount of bicarbonate in the solution, *i.e.* it varied directly as the pH of the solution.

It would appear, therefore, that the reaction of the external medium, as opposed to that of the interior of the cell, is a factor of real importance. In certain cases, however, as in experiments cited in the table,

its effects are covered up by a different factor which favors staining when the interior of the cell is less alkaline. As to the nature of this factor, our experiments are not conclusive but it seems probable that it has to do with the retention of the dye rather than with its penetration.

It must constantly be kept in mind that the intensity with which a cell stains is no index of the penetrating power of the dye in question. A dye may penetrate readily and produce little effect, because it finds nothing within the cell with which to combine, and *vice versa*. In the case of basic dyes the accumulation of large amounts of the dye in the cell sap of plant cells has been attributed by Pfeffer (13) to its combination with tannic acid, while Nirenstein (14) has recently shown that the capacity of neutral fats to take up basic dyes is greatly increased by the presence of small amounts of fatty acids. If in the material used in our experiments the *accumulation* of the dye depended on its combination with some acid constituent of the protoplasm, then it is easy to see why the presence of ammonia which penetrates the cell should hinder staining more than that of NaOH which does not, and conversely why, other things being equal, CO_2 should if anything, favor staining as compared with HCl. This is, however, offered merely as a tentative suggestion.

The general result of these experiments is to show that, other things being equal, as demanded by the first theory, alkalinity of the external medium favors, acidity hinders, the staining of cells by basic dyes. On the other hand, alkalinity of the cell itself, in our experiments at least, hinders staining—a result which is directly opposed to the second theory and which neither supports nor opposes the first theory, but which probably indicates the existence of an additional factor concerned in the storage of the dye. This factor may possibly be some substance or substances of acid nature with which the dye forms a salt-like combination.

SUMMARY.

1. Solutions containing NH_4OH and NaOH, and CO_2 and HCl may be used to produce various combinations of extracellular and intracellular reactions in starfish eggs, *Gonionemus*, and *Nitella* cells.

2. Staining by basic dyes is, with a constant intracellular reaction, favored by increased extracellular alkalinity. With a constant extracellular reaction, staining is hindered by increased intracellular alkalinity.

3. These facts are in opposition to the view that staining of cells by basic dyes is chiefly governed by a combination of the dyes with cell proteins. It is more in harmony with the view that the combination is with a substance or substances of acid nature.

We desire to express our appreciation to Dr. M. H. Jacobs for suggesting this problem to us, and for his advice during the progress of this work.

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THE OXIDATION OF SODIUM LACTATE BY HYDROGEN PEROXIDE.

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(From the Department of Physiology, Harvard School of Public Health, Boston.)

(Received for publication, March 1, 1924.)

The consideration of the manner in which various metabolites are removed from the living cell by the process of oxidation presents a problem which, if it is to be considered quantitatively, necessitates a detailed knowledge of the course of comparable reactions *in vitro*. It is, therefore, desirable to consider such reactions from many points of view before attempting to analyze the more complicated reactions that are associated with the living tissue.

Starting with the admirable researches of Fletcher (1) increasing importance has been attached to variations in the concentration of lactic acid in the animal body. There is, however, very little known about the manner in which lactic acid disappears after its production during muscular contraction. The conditions regarding this phase of muscle physiology have recently been reviewed by Shaffer (2), who points out that there are four ways in which lactic acid may disappear in the animal body. There is the possibility that there may be a coupled reaction, part of the lactic acid being oxidized to provide energy to reconvert the remainder to glucose. Again, there may be a conversion of all the lactic acid to glucose, the oxidation of some other metabolite producing this energy. The third possibility is that part of each molecule of the lactic acid is oxidized with a production of acetaldehyde, which in turn is converted into glucose. Finally, there is the possibility that the lactic acid is entirely burned.

In view of these manifold possibilities a knowledge of the changes *in vitro* is desirable. Such reactions were first studied, in the case of lactic acid, by Fenton (3) who found that there was a slow oxidation of lactic acid by H_2O_2. Later the problem was taken up by Dakin (4) who investigated it in much greater detail. In both researches attention was directed toward the various products of the reaction rather than to the manner in which the reaction took place.

It has been shown by the writer, in a previous paper (5), that it is possible to follow the course of the oxidation of an organic acid by means of determinations of the rate of production of CO_2, as carried out in the apparatus designed by Osterhout for this purpose. The work reported here is an attempt to trace by means of this method the mechanism of the oxidation of sodium lactate by H_2O_2.

The modification of the Osterhout apparatus devised by Irwin (6) was used in these experiments. It was arranged in a brass frame in such a way that it could be lowered into a constant temperature bath. The illumination of the tubes was provided by a 150 watt Daylight lamp placed opposite a window in the bath, so that by means of a mirror and a tube observations could be made without disturbing the apparatus. It was found that this method of reading the indicator tubes is much easier than when the tubes are suspended in air since in the bath there are no high lights and the tubes appear perfectly flat. The temperature maintained in these experiments was 25°C. ± 0.1°C.

Early in the work the desirability of calibrating the apparatus became apparent. The method described by Parker (7) is not adaptable to the Irwin type of apparatus owing to the impossibility of letting in CO_2, but an application of the mass law was made which proved to be useful.[1] In order to apply this method of calibration it is necessary to assume that there is an even distribution of CO_2 throughout the apparatus at any given time. If this assumption is made, then it is possible to compute the milligrams of CO_2 needed to change the colors in the indicator tube between the standards which in this case had the values pH 7.78 and 7.36. It is also necessary that the indicator of the apparatus contain a solution of $NaHCO_3$ of known concentration. The method is as follows:

$$\frac{[\overset{+}{H}]\,[\overset{-}{HCO_3}]}{[H_2CO_3]} = k_a \qquad (1)$$

in which k_a = the dissociation constant of H_2CO_3.

$$[\overset{-}{HCO_3}] = [NaHCO_3]\gamma \qquad (2)$$

[1] The writer wishes to express his indebtedness to Dr. E. J. Cohn for the suggestion of this method and for his assistance in its development.

in which γ is the dissociation constant of $NaHCO_3$.

$$[H_2CO_3] = [CO_2]\,k \tag{3}$$

in which k is the absorption coefficient of CO_2.
From 1, 2, and 3

$$\frac{[H^+]\,[NaHCO_3]}{[CO_2]\,k} = \frac{k_a}{\gamma} \tag{4}$$

or

$$\frac{[H^+]}{[CO_2]} = \frac{k_a}{\gamma} = \frac{k}{NaHCO_3} \tag{5}$$

Equation (5) was used to calculate the concentration of CO_2 at pH 7.78 and 7.36. The value of k_a used was the one given by Kendall, 3.5×10^{-7}; and the value of γ, since the concentration of the $NaHCO_3$ was M/10,000, was taken as 1. The absorption coefficient of CO_2 was also taken as 1.

For pH 7.78 ($[H^+] = 0.167 \times 10^{-7}$)

$$\frac{0.167 \times 10^{-7}}{[CO_2]} = \frac{3.5 \times 10^{-7}}{0.0000841}$$

$$[CO_2] = 0.004 \text{ mg. in 10 cc.}$$

For pH 7.36 ($[H^+] = 0.44 \times 10^{-7}$)

$$\frac{0.44 \times 10^{-7}}{[CO_2]} = \frac{3.5 \times 10^{-7}}{0.0000841}$$

$$[CO_2] = 0.0106 \text{ mg. in 10 cc.}$$

Hence

$0.0106 - 0.004 = 0.0066$ mg. CO_2 to change 10 cc. from pH 7.78 to 7.36.

The volume of the apparatus involved was found to be 90 cc., therefore the number of milligrams of CO_2 necessary to change the indicator between the standards is 0.0594 mg.

Knowing the amount of CO_2 needed for a unit change of the indicator it is a very simple matter to evaluate $\frac{\Delta CO_2}{\Delta T}$ in which T is unit

time. From this the average velocity of the reaction can be determined, which in turn forms the basis of the integral curve.

The sodium lactate used in these experiments was *i*-lactic acid to which NaOH was added until the reaction of the solution was pH 6.9; the concentration of the lactate was M/50. The H_2O_2 was used in molar solution and the reaction was adjusted to the same point as the lactate. It is necessary that care be taken not to use alkaline solutions in the reaction flask of the Osterhout apparatus since any buffer action at this point tends to vitiate the results.

In all the experiments reported the ratio of the peroxide to the lactate is the same, *i.e.* 25 cc. of the lactate to 10 cc. of the peroxide.

The first experiments were on the reaction between sodium lactate and H_2O_2 without a catalyst. Fig. 1 shows the points found experimentally for the rate of production of CO_2. It will be seen that the rate falls off from the start in such a manner that at the end of 2 hours it is very slow. At this point the readings were stopped since, when the time of change of the indicator tube is over 15 minutes, the accuracy of the method is impaired.

The total amount of CO_2 produced in the reaction is given in Fig. 2, this curve being the integral of the rate curve in Fig. 1. There is a slight lag in the curve at the start, the importance of which will be pointed out in conjunction with other experiments.

The next step in this series of experiments was the determination of the amount of H_2O_2 consumed in this reaction. The method used was that of Kingzett, which is more applicable for a solution such as is dealt with here than the permanganate method, since the former is not influenced by the presence of organic matter. It consists in determining the amount of iodine released by the peroxide from KI in the presence of H_2SO_4.

A mixture of the reagents was made up as in the experiments on the production of CO_2, but in larger quantities. A current of air free from CO_2 was passed through the solution and samples were taken from time to time. The results of these titrations are given in Fig. 3, Curve *A*. Instead of a gradual diminution of the peroxide present it was found that the H_2O_2 (or its equivalent in active oxygen) at first rises and then falls off, indicating that an increase in the oxidative energy of the reaction has taken place. There is but one apparent

explanation for this result; some product or constituent of the reaction is activating the oxygen of the air passed through the reaction mixture. The result of this process is the formation of a peroxide-like substance.

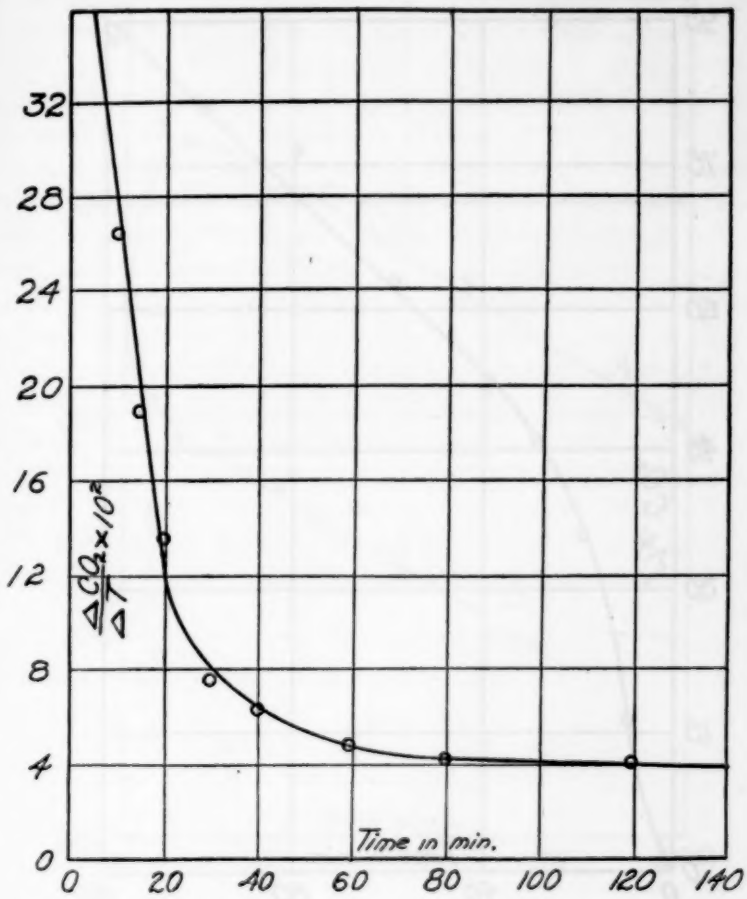

FIG. 1. The rate of production of CO_2 from sodium lactate and H_2O_2. The points represent the average of ten experiments.

If this explanation is correct the increase in oxidative activity that is found in the presence of air should not appear when the reaction is carried out in an atmosphere that is free from oxygen. For this reason experiments were made in which nitrogen instead of air was

passed through the solution, the results being given in Curve *B* of Fig. 3. There is no increase in this case; on the contrary the oxidative activity of the solution decreases from the start.

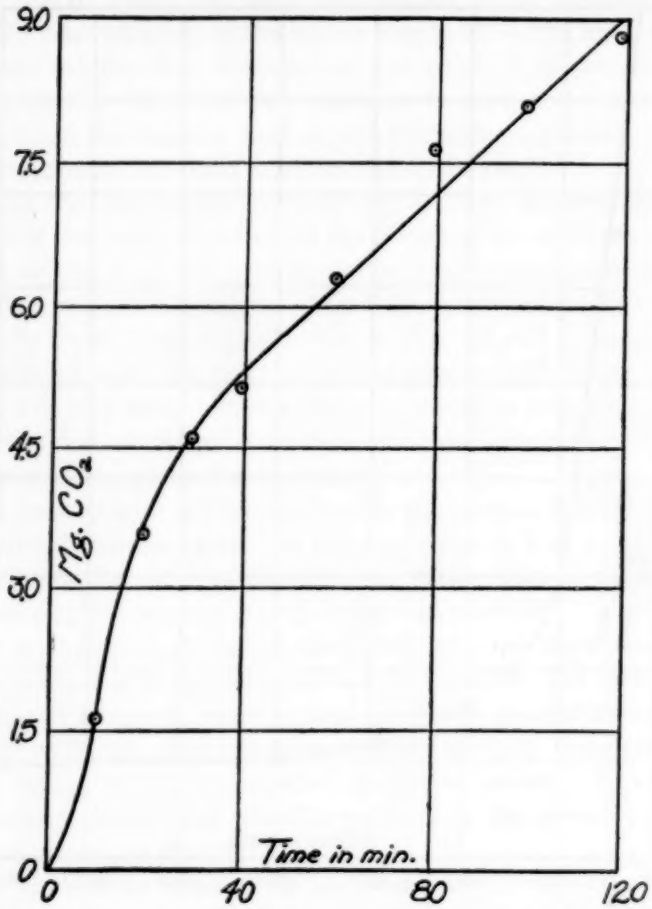

FIG. 2. The total CO_2 produced during the reaction between the lactate and the peroxide. (From Fig. 1.)

The question at once arises as to what constituent of the reaction brings about the increased activity denoted by the rise in Curve *A* of Fig. 3. Dakin (4) points out that among the products of the reaction between ammonium lactate and H_2O_2 is acetaldehyde. This

compound is known to be particularly active in the formation of a peroxide, acetyl peroxide, when allowed to react with oxygen.[2]

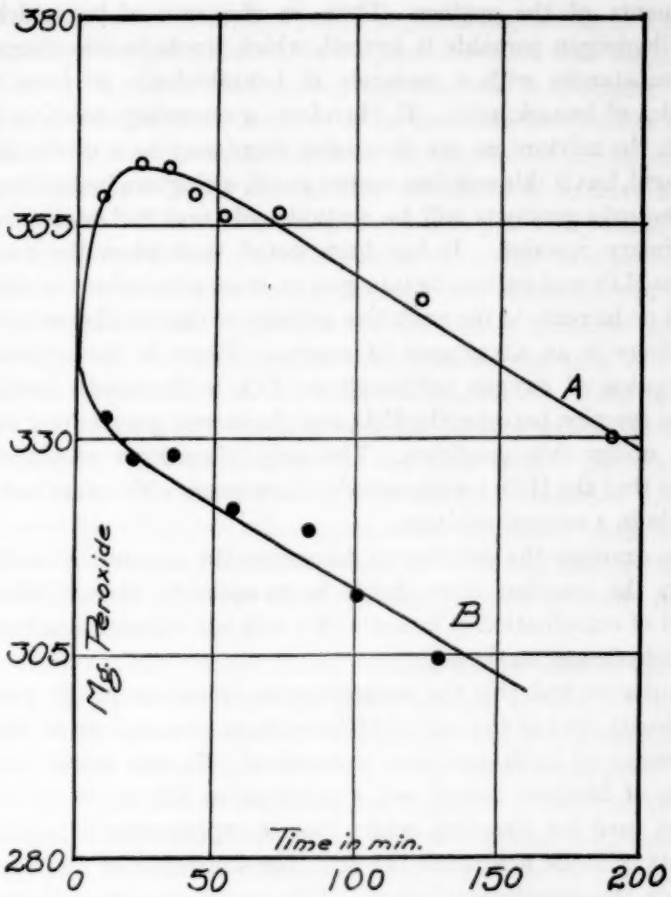

FIG. 3. The changes in the oxidative activity of the peroxide-lactate reaction. For convenience the changes in the oxidative activity of the solution have been plotted in terms of H_2O_2. Curve *A* is the reaction in the presence of air free from CO_2; Curve *B* is the reaction in an atmosphere of nitrogen.

[2] The writer has found that the formation of a substance which acts like peroxide in a solution containing acetaldehyde and oxygen, proceeds more rapidly when H_2O_2 is added.

The formation of such peroxides has been discussed in detail by Baeyer and Villiger (8) who have shown that aldehydes readily form compounds of this nature which, in turn, react with other oxidizable constituents of the system. Thus, in the case of benzaldehyde, benzoylhydrogen peroxide is formed, which reacts in the absence of other substances with a molecule of benzaldehyde to form three molecules of benzoic acid. If, therefore, a secondary reaction takes place in the mixture we are discussing there may be a production of acetic acid, but if this reaction cannot go on, owing to a lack of oxygen, then the only products will be acetaldehyde and carbonates due to the primary reaction. It has been noted that when the reaction between H_2O_2 and sodium lactate goes on in an atmosphere of nitrogen there is no increase in the oxidative activity of the solution as is found when there is an abundance of oxygen. There is, nevertheless, a consumption of oxygen (although no CO_2 is liberated), indicating that the reaction between the H_2O_2 and the lactate goes on to a certain degree under this condition. The only alternative would be to suppose that the H_2O_2 spontaneously decomposes which does not seem probable in a neutral solution.

If we examine the solution to determine the amount of lactic acid used in the reaction there should be no apparent change, since the method of examination (Clausen's (9)) will not differentiate between acetaldehyde and lactic acid.

In order to test this the reagents were mixed as in the previous experiments, and at the end of $3\frac{1}{2}$ hours in an atmosphere of nitrogen the amount of lactic acid was determined. It was found that the amount of bisulfite bound was equivalent to 220 cc. of the iodine solution used for titrating, while control experiments of equivalent amounts of lactic acid used 225 cc. The difference of 2.27 per cent is within the experimental error. This would seem to indicate that the reaction does not go beyond the stage of acetaldehyde unless free oxygen is present. These results are quite in accord with those of Dakin, who found that the first distillation gave practically nothing but aldehyde.

Since we know the amount of oxygen consumed in the reaction it should be possible to calculate how much acetaldehyde is formed from time to time when no atmospheric oxygen is present and, from this,

the probable amount of organic peroxide formed in the secondary reaction, if the acetaldehyde alone is concerned with the formation of this peroxide and the reaction goes on as already outlined. When such a calculation is made, however, it is found impossible to correlate the amount of oxygen activated in the presence of air with the amount of aldehyde that is formed during the reaction in nitrogen. It is plausible to consider that the increase in the oxidative activity of the solution in the presence of air is due to the reaction of acetyl peroxide with sodium lactate rather than with acetaldehyde as we have heretofore considered. This reaction might well take place very rapidly and result in the conversion of practically all of the lactate to aldehyde in a short time. Such an hypothesis, although not susceptible of proof, is quite attractive since it explains the increase in oxidative activity at the start. If the secondary reaction goes slowly, as it well may, then the slow production of CO_2 will be accounted for, since the CO_2 released by the primary reaction with the lactate would be bound immediately by the base liberated at the same time. There can be no liberation of CO_2 from the solution until the reaction between acetyl peroxide and some other constituent has taken place with a production of acetic acid from the peroxide. The fact that the liberation of CO_2 is dependent on the secondary reaction accounts for the shape of the curve in Fig. 2.

That there are carbonates in the solution makes possible the consideration that perhaps there are percarbonates formed during the reaction. A test was made for the presence of percarbonates by adding a solution of $AgNO_3$ to a sample of the reaction mixture. The dense precipitate was centrifugalized and washed. It was then treated with KI and H_2SO_4 and shaken with a small amount of chloroform. There was no perceptible coloration of the globule of chloroform, hence it is assumed that there is no percarbonate formed during the reaction.

Certain experiments were carried out to determine the effect of iron on the amount of CO_2 produced in this system and also its effect on the secondary reactions taking place. The salt used was Fe_2Cl_6 in a concentration of M/10, and the experiments were conducted in two series. In the first group the iron was added to the peroxide before the mixture was added to the lactate solution, and in the second

group the iron was added to the lactate before the peroxide. This variation in the type of experiment was made in order to determine if there was any difference to be noted when the iron was in a highly oxidized state and when it was in the reduced condition. The experiments were carried out in the same manner as those of the first series with the exception that to each mixture there was added 0.05 cc. of iron solution. The results of the first group of experiments are given in Fig. 4, Curve *A*. This is the integral curve of the reading on the Osterhout apparatus and, by a comparison with the curve found when no iron is present (Fig. 4, Curve *B*), there is noted a decided increase in the amount of CO_2 produced.

The results of the second group of experiments of this series are given in Fig. 5, Curve *A*. There is a decided lag in the curve at the start, but following this period there is a considerable production of CO_2 so that in the end the amount produced is practically the same as when the iron is not present (Fig. 5, Curve *B*).

Comparison of the two curves on the effect of iron shows that the condition of this element is of importance in the reaction, especially at the start. This is far more strikingly shown in the following experiments on the disappearance of H_2O_2.

The procedure in these tests was the same as in the preceding determinations of H_2O_2. The iron was added in amounts equivalent to those used in the experiments on the production of CO_2. The results are given in Fig. 6. Curve *A* shows the amount of peroxide used up in the reaction if the iron is added at the same time as the peroxide. There is not, as will be observed, any great difference between this reaction and the one that takes place when no iron is present (*cf.* Fig. 3). As might be expected there is a greater utilization of the peroxide, but this is all. Curve *B* summarizes the second type of experiment with iron. In this case the amount of H_2O_2 (or its equivalent) instead of increasing at the start decreases; this decrease is later followed by an increase. The cause of the decrease is readily determined by an observation of the variations in acidity that occur during the reaction. When the iron is added to the sodium lactate solution there is a production of acid so that the pH falls from nearly neutral to below 5.0. This is due to the reduction of the ferric salt to the ferrous with a production of acid. When the H_2O_2 is added

to the solution the iron is again oxidized and the acidity comes back to the original point. These changes in acidity coincide in point of time with the changes that are noted in regard to oxidative activity.

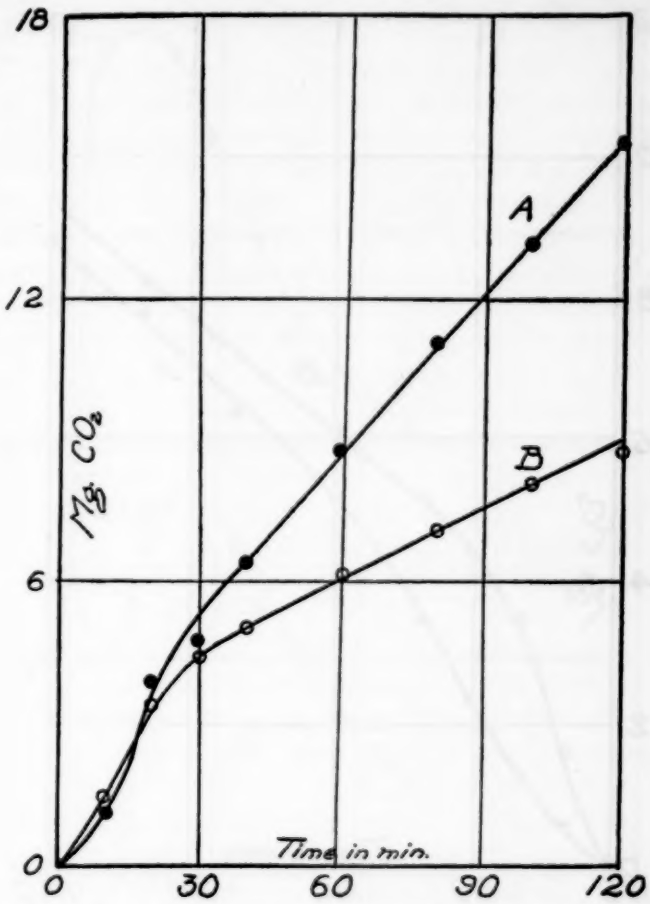

FIG. 4. The effect of iron on the amount of CO_2 produced during the reaction between sodium lactate and H_2O_2 when the iron and the peroxide are mixed before addition to the lactate. Curve *A* is the reaction with iron; Curve *B* is the reaction without iron.

The fact that iron itself is reduced would indicate that the action of this metal in the process of catalyzing oxidations is the oxidation of the iron itself, as has been suggested by Baudisch (10), and by Haber

(11) (*cf.* Mellor (12)). These authors have considered that there is in all probability a formation of a complex between the actor, which in our case is the peroxide, and the inductor, the iron. Baudisch

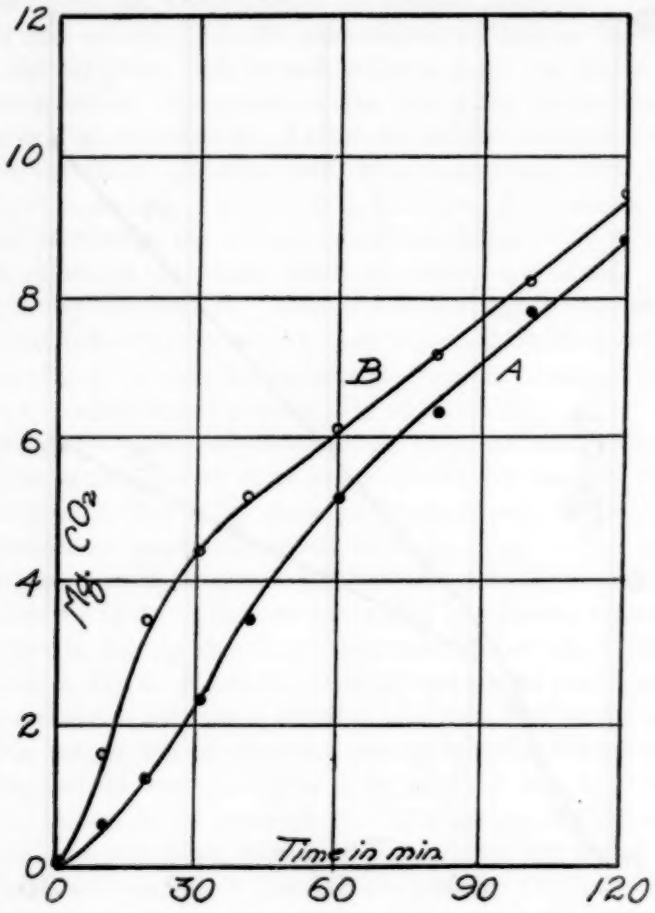

FIG. 5. The effect of iron on the amount of CO_2 produced when the iron is added to the lactate before the addition of the peroxide. Curve *A* is the reaction when iron is present; Curve *B* is the reaction when iron is not present.

developed a theory that, on the basis of Werner's coordination hypothesis, it is possible to consider that the iron salt may form the nucleus of a coordination compound. This hypothetical compound

may be conceived to be of high oxidative energy which is available for the oxidation of other compounds. In the experiments that we are discussing it is quite possible that the iron, when it is added to the

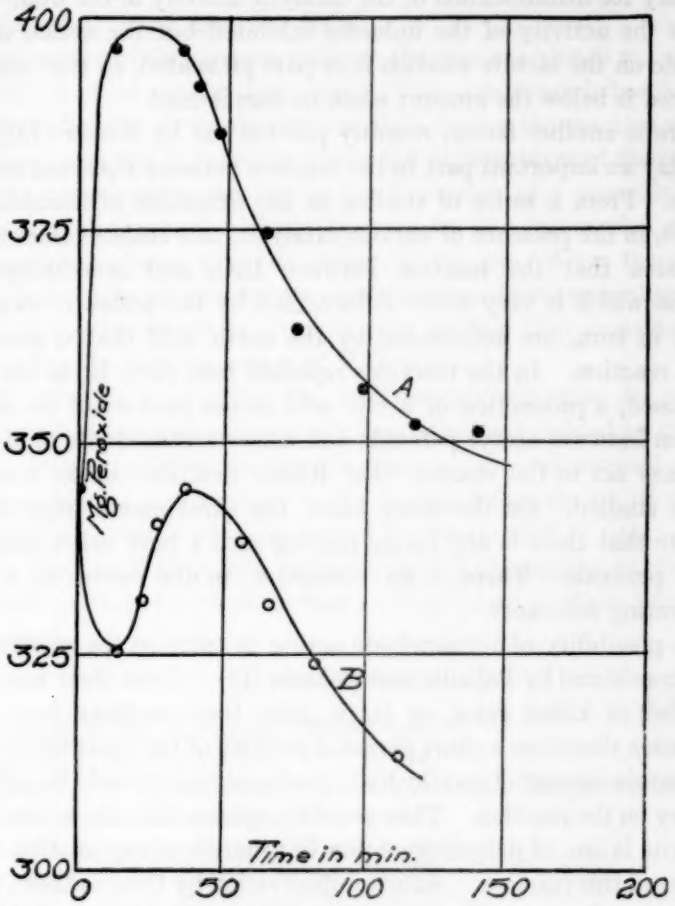

FIG. 6. The effect of iron in different states on the oxidative activity of the reaction. Curve *A* is the result with oxidized iron; Curve *B* is the result with reduced iron.

peroxide, forms just such a compound as Baudisch pictures. This would account for the activity that is shown by the increased production of CO_2. On the other hand, when the iron is added to the lactate

solution there is an immediate reduction which, as has been mentioned, results in a production of acid. Such a condition may well be unfavorable for the production of the complex which can be considered necessary for manifestation of the catalytic activity of the iron. Not only is the activity of the inductor inhibited but the action of the peroxide on the lactate solution is in part prevented, so that the CO_2 produced is below the amount when no iron is used.

There is another factor, recently pointed out by Reiner (13), that may play an important part in the reaction between H_2O_2 and sodium lactate. From a series of studies on the oxidation of acetaldehyde by H_2O_2 in the presence of various catalysts, this author comes to the conclusion that the reaction between H_2O_2 and acetaldehyde—a reaction which is very slow—is increased by the action of catalysts which, in turn, are accelerated by the acetic acid that is produced in the reaction. In the reactions reported here there is, as has been mentioned, a production of acetic acid as the product of the second reaction between acetyl peroxide and either acetaldehyde or lactate. This may act in the manner that Reiner describes in the reactions he has studied. On the other hand, the experimental data do not indicate that there is any factor playing such a part other than the acetyl peroxide. There is no indication in the curves of a third accelerating substance.

The possibility of acetaldehyde acting to carry on the reaction has been considered by Palladin and Sabinin (14). From their results on the effect of killed yeast on lactic acid, these authors came to the conclusion that after a short period of activity of the yeast there would be a certain amount of acetaldehyde produced which would be sufficient to carry on the reaction. They are of the opinion that the action of the aldehyde is one of dehydrogenation but there is no explanation of the manner of this reaction. Another observation by these authors, which is of interest in connection with other statements made here, is the fact that the acetaldehyde is not converted into alcohol in their experiments, which they explain as being due to the large excess of hydrogen acceptor present in the reaction mixture. In our experiments upon the oxidation of lactic acid there is always an excess of H_2O_2 which, on the basis of Palladin's explanation, would account for the fact that we do not find any oxidation beyond the stage of acetaldehyde when the reaction is carried out in an atmosphere of nitrogen.

SUMMARY.

By means of a modification of the technique of the Osterhout apparatus it is possible to follow the production of CO_2 from sodium lactate when acted upon by H_2O_2.

The results of this process indicate that the reaction is not a simple one but is of an autocatalytic type. This conclusion is borne out by the fact that the determinations of H_2O_2 during the reaction show an increased amount of peroxide during the earlier stages of the reaction. This is considered to be due to the formation of a peroxide by the oxidation of the acetaldehyde (formed by the interaction of H_2O_2 and sodium lactate) with the oxygen of the air. When the reaction is carried out in an atmosphere of nitrogen no increase is observed.

Further experiments in nitrogen tend to show that acetaldehyde is the end-product of the action of H_2O_2 alone.

The effect of $FeCl_3$ upon the reaction depends upon the previous treatment of the iron salt. If the iron solution is added to the H_2O_2 before mixing with the lactate there is an increased amount of CO_2. If, however, the iron is added to the lactate before the addition of the peroxide, the action tends to inhibit the production of CO_2.

The reaction of H_2O_2 with sodium lactate is comparable to the action of killed yeast and methylene blue as determined by Palladin and his coworkers.

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THE EFFECT OF CYSTINE AND GLYCOCOLL ON THE OXIDATION OF SODIUM LACTATE BY HYDROGEN PEROXIDE.

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In a previous paper (1) it was pointed out that the mode of action of H_2O_2 on sodium lactate is probably one of dehydrogenation. It was noted that the rate of production of CO_2 was not constant but gradually fell away until, at the end of about 2 hours, it was so slow as to impair the accuracy of the method of determination. The addition of an iron salt ($FeCl_3$), although increasing the total amount of CO_2 produced during the period in which the reaction was studied, does not prevent a decrease in the rate of production of CO_2.

In this paper the results of other means taken to maintain the reaction at a constant rate are reported. If the function of the peroxide is the removal of hydrogen from the substrate, is it not possible that the rate of CO_2 production can be maintained at a constant high level by the addition of some substance similar in nature to those substances known to bring about dehydrogenations in the cell? Thunberg (2) has demonstrated quite clearly that there are certain substances in the cell whose function is the transport of hydrogen. The active group of these substances is, without doubt, the sulfhydryl group. This conclusion has been borne out by Meyerhof (3), who has shown that there is a direct relationship between the intensity of the nitroprusside reaction of yeast extract and the ability of this extract to restore the respiration of washed yeast. Furthermore, Meyerhof found that it was possible to duplicate this action by the addition of thioglycollic or thiolactic acid. The more recent work of Hopkins (4) has resulted in the isolation of a substance whose activity is due to its sulfhydryl group and which is perhaps the *Atmungskörper* of Meyerhof.

In view of these facts it seemed possible that the addition of a substance containing a sulfhydryl group to the mixture of H_2O_2 and sodium lactate would increase the rate of production of CO_2. For this reason experiments were carried out in which a small amount of cystine was added to the reaction mixture. While cysteine is perhaps more desirable as a source of the sulfhydryl group, the stable cystine is more convenient to handle. In either case it is probable that an equilibrium between the reduced and the oxidized forms would occur in a very short time. The concentration of the reagents was the same as in the previous experiments, 0.02 M sodium lactate and M H_2O_2. The cystine was made up in a suspension, 1 cc. of which represented 5 mg. of the amino acid. The rate of production of CO_2 was determined by a modification of the Osterhout apparatus in the manner previously described (1).

The experimental procedure consisted in placing 25 cc. of the lactate solution in the reaction flask of the apparatus and passing a current of air free from CO_2 through the solution for 1 hour. At the end of this time the H_2O_2 (10 cc.) and 5 mg. of cystine were added, and the rate of production of CO_2 was determined as often as possible. The readings were converted into terms of $\frac{\Delta CO_2}{\Delta T}$, in which form they were plotted giving the curve shown in Fig. 1, Curve *A*. As may be seen, there is a period at the start where the rate of production of CO_2 is identical with the rate when no cystine is present (Fig. 1, Curve *B*). This period is comparatively short and soon the rate increases and holds at a high level for a considerable length of time, after which there is a decrease.

There are certain factors which may play a part in causing the decrease and the subsequent increase in the rate observed in the first few minutes. The first of these is the insolubility of cystine—a fact that must definitely affect a second factor, namely, the conversion of a portion of the cystine to cysteine. In this reaction mixture, which contains not only an oxidizing agent but also a reducing substance, an equilibrium between the cystine and the cysteine must be formed. Until this equilibrium is reached the reaction cannot go on at its maximum velocity.

There is another factor, also, to be considered. In a previous paper (1) attention is called to the fact that when sodium lactate reacts with H_2O_2 acetaldehyde is produced. Wieland (5) has shown that the reduction of methylene blue by milk in the presence of an aldehyde is due to the action of a dehydrase. It is possible that in this case the cystine is playing the part of the dehydrase with the effect that the reaction is very much accelerated.

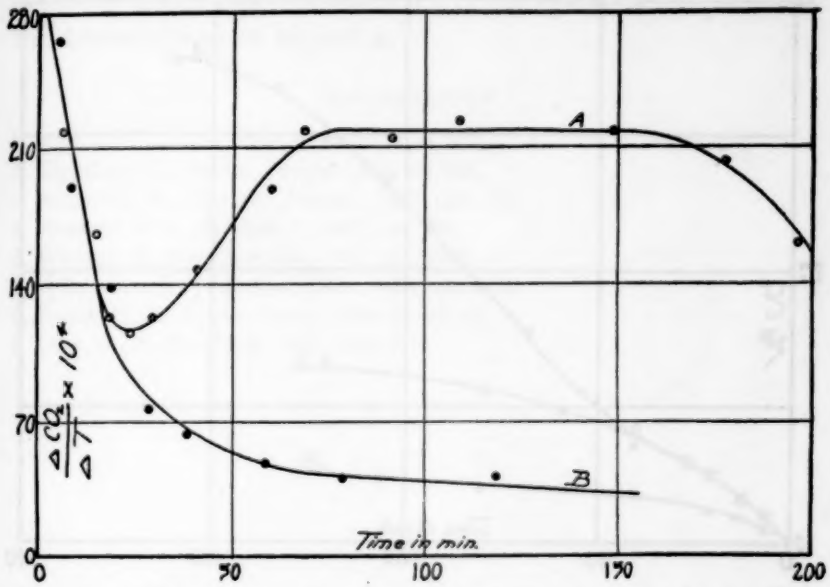

FIG. 1. The effect of cystine on the rate of production of CO_2 from the reaction between sodium lactate and H_2O_2. Curve *A* is the result when cystine is added; Curve *B* is the result when no cystine is present.

The total amount of CO_2 produced is given in Fig. 2. Curve *A* shows the amount produced when cystine is present, while Curve *B* shows the amount produced in the control experiment without cystine. It is quite evident that there is more CO_2 produced than can be accounted for if the cystine were simply oxidized by the H_2O_2. There is, however, the possibility that a certain portion of the amino acid is destroyed in the reaction, thus resulting in the liberation of ammonia.[1] It is important that this point be considered since Witzemann (6)

[1] There is no evidence that sufficient free ammonia is produced to affect the color in the indicator tube.

has shown that ammonia has a catalytic effect on the oxidation of butyric acid by H_2O_2. Furthermore, the presence of an amino group may not be without effect for Dakin (7) has pointed out that some amino acids will catalyze certain organic condensations.

In order to determine the effect of the amino group or the possible production of NH_3 on the reaction between H_2O_2 and sodium lactate, as manifested by the production of CO_2, 5 mg. of glycocoll were added

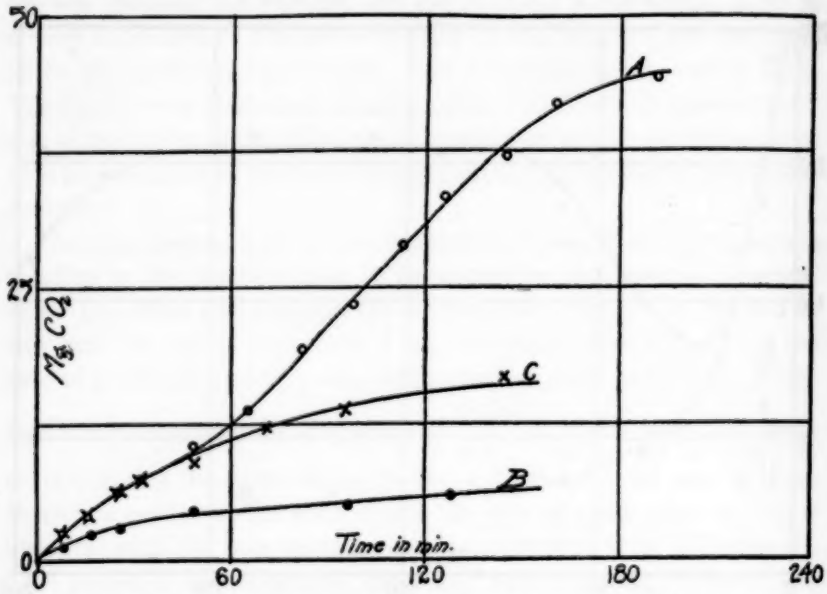

FIG. 2. The effect of cystine and glycocoll on the amount of CO_2 produced by the reaction between sodium lactate and H_2O_2. Curve *A* is the amount produced when cystine is present; Curve *B* is the control curve of H_2O_2 alone; Curve *C* is the effect of glycocoll.

in the same manner as the cystine in the preceding experiments. The results are given in Fig. 2, Curve *C*. There is no doubt that there is a definite effect due to the presence of the glycocoll; it is not, however, of the same order as the effect resulting from the addition of cystine. The effect is quite what one might expect from the results of Dakin (7) and of Witzemann (6). Furthermore, these results are comparable to the effect of glycocoll on dogs, as determined by Lusk (8), who found that small doses increased the heat production.

SUMMARY.

The rate of production of CO_2 from sodium lactate when treated with H_2O_2 may be increased by the addition of a compound containing a sulfhydryl group, *i.e.* cystine.

A small part of this increase in rate of CO_2 is due to the action of the amino group as shown by the action of glycocoll.

The results tend to show that the mode of action of H_2O_2 is one of dehydrogenation and that the action of the cystine is comparable to the *Atmungskörper* of Meyerhof.

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ON STEREOTROPISM IN TENEBRIO LARVÆ.

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I.

Proof of the tropistic character of orientation induced through lateral contact with a surface, in a creeping animal of suitable sensitivity, has been described for *Parajulus* and other diplopods (1). In view of the scant attention heretofore given to stereotropism, it is worth while to extend and to develop the proof in relation to other organisms. The conditions under which stereotropism suffers reversal of sign also deserve careful scrutiny for the sake of comparison with the known reversals of behavior in other tropistic responses.

With the diplopods it was shown that posterior unilateral contact of the creeping animal with the edge of a thick glass plate forces the head to turn in the direction of the contact. The response persists only so long as the contact is maintained. It may at any moment be brought to an end by removing the glass plate; the homostrophic response (1) due to the previously established bending of the body, immediately supervenes. Moreover, if equal areas of contact obtain on the two sides of the animal stereotropic bending fails to appear.

Precisely similar phenomena are observed with the meal worm larvæ of *Tenebrio*. Earlier attempts (1) to study homostrophy in these insects were not very successful, owing to the general increase in tone of the abdominal musculature which results from local mechanical stimulation in attempts to bend the tail of the larva to one side. Homostrophic response is easily obtained, however, by allowing the animal to creep past the corner of a thick glass plate with whose edge the larva has been in lateral contact. The observations are made in a dark room, under red light of low intensity. Fig. 1 shows the result of such an experiment.

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II.

We are concerned here merely with the facts (*a*) that a true stereotropic orientation results from lateral contact of the moving organism with a surface; and (*b*) that the orientation is dependent upon the continuous generation of stimuli derived from such contact.

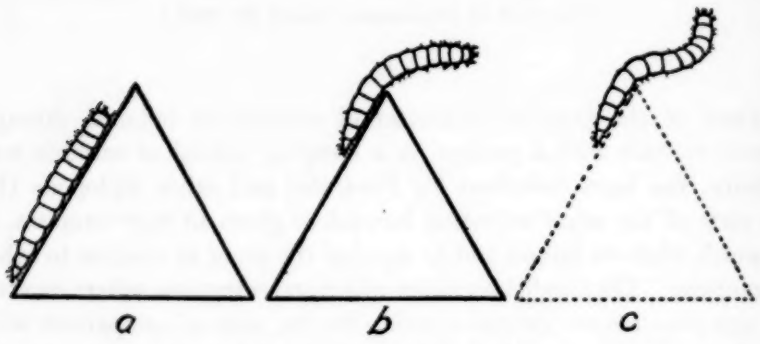

FIG. 1. Stereotropic orientation of larval *Tenebrio* (*a*, *b*, successive positions) and reflex homostrophic orientation released (at *c*) when source of contacts is removed.

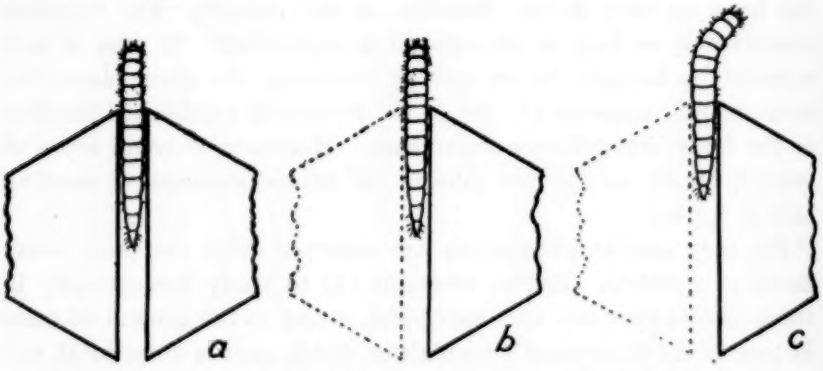

FIG. 2. Balanced action of equal bilateral contact zones shown in pursuit of a straight course (*a*). Removal of contact plate on one side (*b*) is immediately followed by stereotropic bending (*c*).

Where a *Tenebrio* larva creeps between two glass plates so arranged as to give equal contacts on the two sides of the animal, the larva on emergence from the zone of contacts pursues a perfectly straight course. The experimental manipulation is not quite so easy as

with *Parajulus*, since the posterior region of the body of the *Tenebrio* larva tapers, whereas that of *Parajulus* is nearly constant in diameter. But the result, pictured in Fig. 2, *a*, is not especially difficult to observe.

The orienting influence of contact appears also in the tendency of a *Tenebrio* larva to creep over an object with which it is in lateral apposition. This it does by lifting the head and bending it sharply toward the side tactually stimulated, as shown in Fig. 3.

The responses to temporary localized contacts of sufficient area are of some special interest. If the anterior end be momentarily

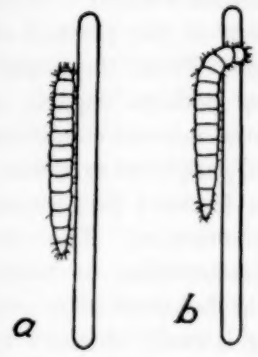

FIG. 3.

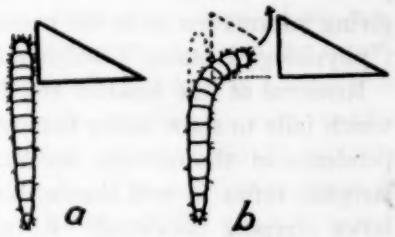

FIG. 4.

FIG. 3. A creeping larva in lateral contact with a glass rod (*a*) elevates the head and swings anterior end of the body toward the source of contacts (*b*).

FIG. 4. Stereotropic response is exhibited (*b*) after the source of contact (*a*) is removed.

touched at one side with the flat edge of a piece of glass and the glass quickly withdrawn, the head bends toward the previously existing contact. This response, illustrated in Fig. 4, is quickly followed by resumption of the originally straight path of creeping.

This particular observation shows how under certain conditions a tropistic orientation may be initiated and continued after the external inciting cause has ceased to act. In this respect the experiment gives a simple model of the phototropic orientation described for the firefly by Mast (2). As Loeb has already pointed out (3), it is of course no contradiction of phototropism doctrine, that orienta-

tion should occur after the stimulating agency has ceased to act. It is sufficient to suppose that either the peripheral sensory disturbance initiated by activation continues for an interval after the outer stimulus is withdrawn; or else, that a central nervous state is induced, in this case by unilateral contact of the head with a solid object, which persists for a detectable interval. The probable correctness of the latter interpretation is suggested by the fact that if the organism be already in a state of stereotropic activity, as in the case where posterior lateral contact is being maintained on one side only (Fig. 5, *a*), the response to a new contact surface on the opposite side of the body (see Fig. 5, *b*) is exhibited without sensible delay, resulting in an immediate readjustment of the position of the head appropriate to bilateral stimulation. From this standpoint the various stereotropic reactions seem perhaps capable of giving information as to the persistence of central nervous conditions ("physiological states") which may result from peripheral excitation.

Removal of the head of the *Tenebrio* larva leaves a preparation which fails to show either homostrophy or stereotropism. The independence of the nervous mechanisms for stereotropism or homostrophic reflex is well shown, however, by the behavior of normal larvæ creeping backward. Reversed creeping is easily obtained by breathing upon a quiescent individual; it may also be induced by illumination of a larva in lateral contact with a glass plate. In reversed creeping stereotropism is shown by the positive curvature of the posterior end of the body as it protrudes beyond a contact plate. Homostrophy is not evidenced in backward creeping.

III.

The stereotropic orientation of *Tenebrio* larvæ readily undergoes reversal of sign. The responses already described are those most usually obtained. But if a larva is creeping very rapidly, as a result of previous handling or repeated mechanical stimulation, it orients away from the edge of the glass plate instead of toward it. This is also true with the diplopods. Negative stereotropism inhibits homostrophy. Temperatures below 14°C. and starvation for several days are also conditions in which negative stereotropism tends to be exhibited.

Such reversal might be due either to a central nervous state connected primarily with the speed of creeping at the moment, or to the altered character of the peripheral stimulation. The tactile excitations responsible for the stereotropism of *Tenebrio* and *Parajulus* are due to the effect of gentle contact upon the body surface and its "hairs." Sufficiently vigorous contacts evoke negative stereotropism. In a slowly creeping larva, not in lateral contact with foreign surfaces, a gentle stroking of the body "hairs" (without touching the body surface directly) results in positive orientation of the head; more vigorous stroking leads to the bending of the head away from the side stimulated.

Reversal of response as determined by relative intensity of stimulation does not necessarily imply duplicity of sensory organs. The contact stimulation of the foot of *Chiton* (4) supplies an instance in point. Contact with a surface area of less than about 25 sq. mm. leads to local retraction of the foot, although, as shown by experiments with the part of the foot temporarily involved in a locomotor wave, positive attachment is physically possible to even smaller areas of contact; contact with a surface greater than 25 sq. mm. leads to positive attachment of the foot. The positive response to contacts sufficiently large is reversed by strychnine (5). It is sufficient, therefore, to conceive that reversal of response is due solely to central phenomena, and that a single type of peripheral receptor is involved in both positive and negative stereotropism (6). Vigorous "self-stimulation" of body hairs during rapid creeping is responsible for negative stereotropism in the arthropods studied, but the same effect may be produced by suitably stimulating the body hairs of a slowly creeping larva with a moving object; hence there is no need to suppose a special central nervous state determined merely by the fact of rapid progression.

IV.

It should be important to quantitate the stereotropic orientation in terms of the relative amounts of excitation on the two sides of the body. Experiments with this end in view have not as yet given precise results. This is due chiefly to the fact that the amount of stereotropic bending due to a unilateral contact depends a good deal

upon the rate of creeping. Qualitatively, however, it is easily shown that while equal amounts of contact on the two sides obliterate the tendency to bend, unequal amounts of contact do so only partially and in proportion to the difference between the areas of contact on the two sides.

Thus in Fig. 5, *a*, the slowly creeping *Tenebrio* larva showing positive curvature with reference to one zone of contact has brought into touch with its opposite side a surface of lesser extent. A new position

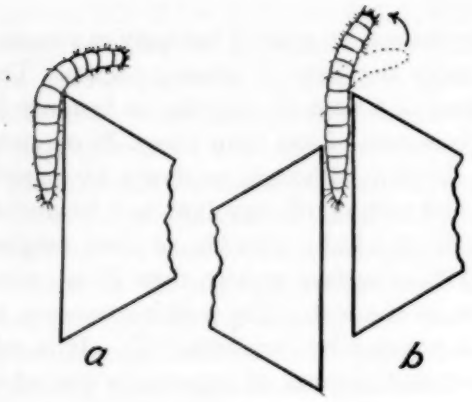

FIG. 5.

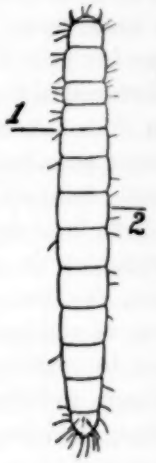

FIG. 6.

FIG. 5. A larva in stereotropic orientation toward unilateral contact (*a*) responds by lesser stereotropic curvature when a less extensive contact is introduced at the other side (*b*).

FIG. 6. Showing asymmetrical distribution of tactile "hairs." Positions of knife edges in experiments discussed in text are indicated at 1 and 2. ×2.4.

of orientation, showing lesser curvature toward the side originally stimulated, is immediately assumed (Fig. 5, *b*).

Interpretation of such experiments requires preliminary proof that the effective stimulation is not derived solely from the level of the body at which contact is no longer maintained. In the experiment illustrated in Fig. 5 it might be suggested that the animal bends most strongly toward that side on which the contact ceases nearer the head. This interpretation would presuppose that the

effective stimulation is local, rather than general over the whole area of contact. But it could not then be understood why when the body is completely in contact along either side the animal should tend to come to rest, which with *Tenebrio* and especially with *Parajulus*, as with *Nereis* (7), is precisely the case. The outcome of tests in which two knife edges at different segmental levels are used to stimulate the body hairs (Fig. 6) is also unfavorable to this view. In general, a larva creeping between two knife edges placed as in Fig. 6, not too far apart, maintains a straight path. The sparse distribution of the "hairs" introduces irregularities of stimulation, however, and a better test is made with two zones of contacts beginning at the same level anteriorly but not of the same length. During ordinary creeping the result of such a test is always a bending of the head toward the side of more extensive contacts.

Experiments made to determine the orienting effect of unequal bilateral contacts were based upon the assumption that equal areas of surface would be equivalent in activating ability. Since the tactile hairs are often if not indeed usually asymmetrical (Fig. 6) in distribution, the assumption could be justified only in case this structural asymmetry is dynamically balanced. There has been in the actual trials every indication that the functional symmetry does not fluctuate with uneven distribution of the body hairs.

V.

The stereotropic response of *Tenebrio* and *Parajulus* is inhibited by light of sufficient intensity. Creeping under red light the animal persistently maintains contact with a vertical glass plate at one side. Horizontal white light of low intensity, coming through the vertical glass, does not influence the stereotropism, though adequately enforcing negative phototropism when no lateral contact is involved. At 16°C. light of about 132 meter candles intensity is required to inhibit stereotropism and to produce orientation away from a contact surface.

Bending of the anterior end toward the source of contact when the head has passed the solid surface (Fig. 1, 2) is also inhibited by light of about 132 m.c.

Photochemical inhibition of stereotropism in the tube feet of starfish was described by Moore (8). In this case an intensity of about 140 m.c., acting for 2 seconds, caused release of the terminal suckers from a glass plate.

It will be noticed that as result of this analysis it becomes possible not merely to "measure one tropism in terms of another" (9, 8), but to predict, or at least to understand, variations in the conduct of *Tenebrio* larva as determined under the simultaneous action of contact, light, temperature among external agencies, and of the homostrophic reflex of proprioceptive origin.

SUMMARY.

Larvæ of *Tenebrio* while creeping show homostrophic responses, and stereotropic orientation to lateral contacts. Homostrophic orientation is inhibited by stereotropism. Both depend upon the anterior portion of the central nervous system. Stereotropic orientation due to unilateral contact, particularly at the anterior end, persists briefly after the cessation of the contact.

Equal posterior bilateral contact of the body obliterates stereotropic bending. Unequal posterior bilateral contacts lead to orientation through an angle roughly proportional to the differences in contact areas. Functional symmetry in such responses is not disturbed by asymmetrical distribution of the body "hairs."

The stereotropic orientation undergoes reversal of direction, central in origin, when the stimulation is sufficiently intense.

Stereotropic response, leading to maintained lateral contact with a surface or to bending when the end of such a surface has been passed, is inhibited by a definite intensity of light.

These findings (1) round out the demonstration that stereotropism is truly of a tropistic character, and (2) make possible the understanding of conduct in a case involving the participation of contact stimulation, phototropism, temperature, and homostrophy.

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THE RELATION BETWEEN THE INITIAL RISE AND THE SUBSEQUENT DECLINE OF MILK SECRETION FOLLOWING PARTURITION.

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(From the Department of Dairy Husbandry, University of Missouri, Columbia.)

(Received for publication, January 28, 1924.)

It is a common observation of dairymen that the milk flow of the dairy cow rises for some time after calving and then declines steadily. In preceding papers[1] we have presented data and have offered an interpretation for the declining segment of the curve of milk secretion. Having found that after the first month of lactation the curve of milk secretion with the advance of the period of lactation may be represented by the exponential law representing the course of monomolecular change in chemistry, we suggested the interpretation that the course of milk secretion is probably limited by a monomolecular chemical reaction. This interpretation seemed reasonable in view of the fact that milk secretion must be a chemical or physicochemical process since certain substances typical of blood are converted into other different substances typical of milk. The initial rise of milk secretion, on which no data were available at that time, was explained as due to the improving condition of the animal and to the increasing food consumption due to the improved condition. However, a comparison of the curve of food consumption with the curve of milk secretion does not show the expected parallelism,[2] and the condition of the animal appears to become normal within a short time after calving which is long before maximum milk secretion is reached. These facts suggest that under favorable conditions of food and management the factors limiting or controlling the characteristic rise and

[1] Brody, S., Ragsdale, A. C., and Turner, C. W., *J. Gen. Physiol.*, 1922–23, v, 441, 777.

[2] Unpublished data of the Missouri Experiment Station compiled by C. W. Turner.

decline in milk secretion are inherent in the mechanism of milk secretion. In other words, milk secretion increases, then decreases, and a superabundant food supply is merely stored as body fat and does not appreciably affect the characteristic course of the curve.

The purpose of this communication is to extend the physicochemical interpretation employed to explain the mechanism of the declining curve of milk secretion so as to include the first month of the lactation period during which time the curves of milk secretion rise and pass through a maximum as shown in Fig. 1.

There are a number of well known processes in chemistry classed under simultaneous consecutive reactions which show in their velocity curves the characteristic rising and declining course exhibited by the curves of milk secretion of Fig. 1.[3] For this purpose we compared the course of the curves of milk secretion with the course of the curves of consecutive reactions in order to interpret the curves of milk secretion from this physicochemical view-point.

As previously pointed out, milk secretion, after the second month, falls off in a geometrical progression with time, and the course of the curve after the second month may, therefore, be represented by the exponential equation

$$M = Ae^{-k_1 t} \tag{1}$$

in which M is the milk flow at the time t and k_1 is the constant of decline or the specific reaction rate. This is the integrated form of the equation representing the law of monomolecular change in chemistry. The differentiated form of equation (1) may be written

$$\frac{dm}{dt} = -km$$

which indicates that the amount of milk secreted at any time, t, is directly proportional to the concentration of the substance or factor, or to the intensity of the condition, which controls or limits the velocity of the process of milk secretion at that time.

Similarly, the first part of the rising segment of the curve of milk secretion may likewise be represented by the equation of monomolecular change.

[3] *Cf.* Lewis, W. C. McC., A system of physical chemistry, London and New York, 1920, 3rd edition, 402-6.

$$M = B\,(1 - e^{-k_2 t}) \qquad (2)$$

in which M is the milk flow at the time t and k_2 is the characteristic constant of decline of the rising curve of milk secretion. It will be observed in Fig. 1 that the rise in milk secretion becomes less and less as it approaches the maximum and the decline in this rise is exponential.

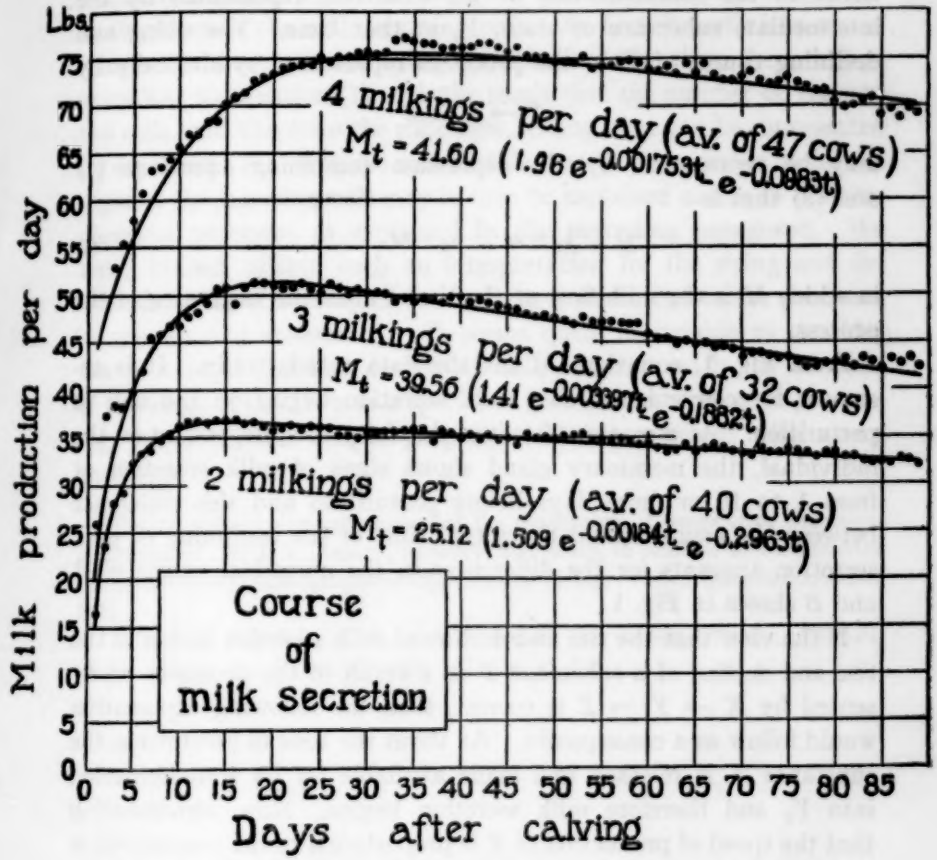
Lbs.
Milk production per day
4 milkings per day (av. of 47 cows)
$M_t = 41.60\ (1.96\,e^{-0.001753t} - e^{-0.0983t})$
3 milkings per day (av. of 32 cows)
$M_t = 39.56\ (1.41\,e^{-0.000397t} - e^{-0.1882t})$
2 milkings per day (av. of 40 cows)
$M_t = 25.12\ (1.509\,e^{-0.00184t} - e^{-0.2963t})$
Course of milk secretion
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85
Days after calving

FIG. 1. The course of milk secretion with the advance of the period of lactation of Holstein-Friesian cows. The curves of milk secretion may be extended to any desired time after parturition by the use of the given formulæ.[1] Data bearing on the causes of the differences in the time of maximum production, differences in the slopes of the curves, and differences in the absolute production of the different groups of cows will be presented later.

The curve of equation (1) declines asymptotically, the curve of equation (2) rises asymptotically. Therefore, the milk secretion at any time appears to be the resultant of two processes, a process $X \rightarrow Y$ which follows the curve represented by equation (2) and a process $Y \rightarrow Z$ which follows the course represented by equation (1), and the velocity of milk secretion at any time appears to be proportional to the concentration, or the condition represented by the intermediate substance or state, Y, at that time. The rising and declining course of Y in the processes represented symbolically by

$$X \rightarrow Y \rightarrow Z$$

may be represented by the expression combining equations (1) and (2) that is

$$M = Ae^{-kt} - Be^{-kt} \tag{3}$$

in which M is the milk flow at the time t after the beginning of the process.

From Fig. 1, equation (3) fits the data satisfactorily. It is assumed, for convenience, that milk secretion begins on the day of parturition. As a matter of fact in the dairy cow, depending on the individual, the mammary gland shows signs of milk secretion at from 1 to 10 or more days before parturition and this difference between the assumed, and the actual time of the beginning of milk secretion accounts for the differences in the numerical values of A and B shown in Fig. 1.

If the view that the rise and decline of milk secretion is due to the rise and decline of a substance Y as a result of the processes represented by $X \rightarrow Y \rightarrow Z$ is correct, then the following explanation would follow as a consequence. At about the time of parturition the substance X is released and made available for its transformation into Y, and therefore milk secretion begins. Now, remembering that the speed of production of Y is proportional to the concentration of X, and the speed of disappearance of Y is proportional to the concentration of Y, it is clear that since at about the time of parturition there is present, by hypothesis, all of X and no Y, the production of Y is greater than its change to Z, and consequently the amount of Y increases and with it there is an increase of the milk flow. As X de-

creases and Y increases the disappearance of Y becomes greater than the production of Y, and so the initial upward course of Y, and of the milk flow, is changed to a downward course as represented in Fig. 1.[4]

Instead of assuming that X and Y are simple chemical substances it may be assumed that the rise and decline in milk secretion following parturition is due to the development and decline in the number or in the vigor of the milk secreting cells. At first the cells increase in number or in vigor according to equation (2), then they decline according to equation (1) with the result that the number or vigor of the cells, and therefore the milk flow, at any time can be represented by equation (3). But the rise and decline in the number or in the vigor of the secreting cells may in turn be explained as due to limiting chemical processes as explained in the preceding paragraph. We have, indeed, offered such an interpretation for the rising and declining course of vitality of the body as a whole due to the processes of growth and senescence.[5] It seems quite reasonable to consider the process of rise and decline of milk secretion with the advance of the stage of lactation as a species of growth and senescence possessing, however, the unique property of being renewed with every gestation.

[4] For an enlightening explanation of the mechanism of consecutive processes, *cf.* Osterhout, W. J. V., Injury, recovery, and death, in relation to conductivity and permeability, Monographs on experimental biology, Philadelphia and London, 1922.

[5] Brody, S., Ragsdale, A. C., and Turner, C. W., *J. Gen. Physiol.*, 1923–24, vi, 31. Brody, S., *J. Gen. Physiol.*, 1923–24, vi, 245.

VALENCY RULE AND ALLEGED HOFMEISTER SERIES IN THE COLLOIDAL BEHAVIOR OF PROTEINS.*

III. The Influence of Salts on Osmotic Pressure, Membrane Potentials, and Swelling of Sodium Gelatinate.

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(Received for publication, February 8, 1924.)

INTRODUCTION.

Recent investigations of the physicochemical properties of proteins have established the fact that proteins are amphoteric electrolytes which can give rise to both positive and negative protein ions. The positive protein ions may combine with negative acid ions to give rise to protein-acid salts, while the negative protein ions may combine with positive metal ions forming metal-protein salts. The formation of one or the other protein product depends on the pH of the aqueous solution of protein, since the pH determines the number of positive and negative protein ions in solution, and whether the positive or the negative ions are in excess. At a definite pH for every individual protein, called the isoelectric point, the number of positive protein ions equals the number of negative protein ions and the protein then does not combine either with acids or with bases. The total degree of ionization of the protein at this pH is shown[1] to be a minimum, and the protein may be said to be almost non-ionogenic.

Thus it is possible to say that protein can occur in three states, which are defined by their hydrogen ion concentrations; namely, (*a*) as non-ionogenic or isoelectric protein, (*b*) as protein-acid salt, such as protein chloride, protein sulfate, etc., and (*c*) as the metal salt of protein, such as sodium or calcium proteinate.

* Parts I and II, *J. Gen. Physiol.*, 1922–23, v, 665, 693.

[1] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922, i, 57.

The effect of various electrolytes on the physicochemical properties of proteins varies with the state in which the protein exists—whether it is isoelectric or whether it exists in one of the other two states. In general it is possible to say that the relation between proteins and ordinary electrolytes depends on the electrical charge of those protein ions which are in excess. This is especially true when we are dealing with those physicochemical properties which are regulated by Donnan's law of membrane equilibria, such as osmotic pressure, membrane potentials, and swelling. Salts in general have little effect on the physicochemical properties of isoelectric protein, as was shown repeatedly by Loeb[2] in his former work, while the addition of a salt to a solution of protein which exists in one of the other two states causes a depression in the magnitude of such phenomena as osmotic pressure, swelling, etc. But the amount of depression varies with the state of the protein and the character of the ions of the added salt. It has been generally established by Loeb that when an electrolyte, such as a neutral salt, is added to an aqueous solution of protein, of the two kinds of ions into which the salt is ionized, only those ions which carry a charge opposite to that carried by the majority of the protein ions have any influence on the above mentioned properties of proteins. This is also true with respect to acids or bases, except that an acid or base may change the pH of the protein and thus produce a change in the degree of ionization of the protein or even a change in the state in which the protein exists, *i.e.* changing it, for example, from isoelectric protein to a protein-acid salt in one case or to a metal-protein salt in the other case.

Another factor which influences the depressing effect of salts on the said physicochemical properties of proteins is the valency of those ions which carry opposite charges to those of the protein ions. It was found that the higher their valency the more effective are those ions in causing a depression in the magnitude of the above mentioned properties of proteins. Thus it was found in the second publication of this series that when salts were added to gelatin chloride—where the gelatin ions were positively charged—of the various salts used only those salts that contained anions of the same valency exerted an identi-

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

cal influence on the osmotic pressure, P.D., and swelling of gelatin no matter what the valency of the cations was; while salts containing the same kind of cations but anions of various valencies exerted influences in agreement with the valencies of the anions.

The following work is an extensive study of the effect of various salts on the same properties of protein when the protein exists in the state of a sodium-protein salt. It was thought desirable to undertake this study not only in order to make the investigation more complete, but also because of the importance of this phase of the problem from the physiological point of view, since the proteins existing in the mammalian organism are in most cases found as metal-protein salts such as sodium, potassium, or calcium salts. Gelatin was again selected as the most convenient protein for an investigation of the type dealt with in this paper, since it is easily obtained in the market in powdered form and can readily be made salt-free. Besides, gelatin shows better than any other protein such a "colloidal" behavior as swelling, which is one of the properties dealt with in this study.

Effect of Salts on the Osmotic Pressure of Sodium Gelatinate.

The general method described in the first paper for measuring the osmotic pressure was also used in this work except for the extra precautions which had to be taken in order to prevent the access of CO_2 from the air to the solutions during the experiment.

It was preferred to use gelatin of pH 11.0, since it was found by Loeb[3] that gelatin gives in the neighborhood of this pH its maximum of osmotic pressure. Besides, gelatin of pH 11.0 as well as salt solutions of the same pH are less liable, in the absence of other buffer solution, to change their pH through slight contamination with CO_2 than at a lower pH. On the other hand, gelatin of a pH higher than 11.0 becomes rapidly hydrolyzed and decomposed. There was no difficulty in bringing all the salts used for this study to pH 11.0 by means of NaOH solution. In most cases they required practically the same amount of NaOH as it was necessary to add to pure distilled water in order to bring it to pH 11.0, which shows that at pH 11.0 all salts cease to act like buffers.

[3] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85, 547.

A stock of a concentrated solution of isoelectric gelatin was prepared from Cooper's non-bleached powdered gelatin by the method described by Loeb[2] except that the gelatin, after it had been soaked for 1 hour in cold M/128 acetic acid, was washed with cold H_2O of pH 4.7, as suggested by A. M. Field.[4] The washed gelatin was melted and its concentration (13.5 per cent) was determined by evaporating a definite volume of it in an electric oven at 100°C. for 24 hours. The stock solution of isoelectric gelatin was distributed into a number of small Pyrex flasks, each flask containing enough of the isoelectric gelatin for a series of about fifteen solutions. This was done in order to avoid the necessity of reheating repeatedly the same gelatin as would be the case if the total stock were kept in a single vessel. The flasks were stored in a refrigerator at about 3°C. When an experiment was made, a single flask containing the concentrated isoelectric gelatin was then removed from the refrigerator and the gelatin was liquefied by immersing the flask in warm water of not higher than 50°C. until the gelatin became very fluid. The gelatin was then made up to a 2 per cent solution of pH 11.0 through addition of H_2O and the necessary amount of NaOH. This happened to be 16 cc. of 0.1 N NaOH per 100 cc. of 2 per cent gelatin solution, as determined by electrometric titration.

A series of solutions of 50 cc. each of various concentrations of the salt to be used was prepared by bringing a half molar solution of the salt to pH 11.0 by means of NaOH and then diluting it to the various dilutions by means of H_2O of pH 11.0. These solutions were mixed with equal volumes of the 2 per cent gelatin solution of pH 11.0, thus giving a series of 1 per cent solutions of gelatin containing various concentrations of a salt of the same pH. These solutions were used as the inside solutions (inside of collodion membranes) for the osmotic pressure measurements.

For the outside solutions a series of salt solutions of 450 cc. each was made up in the same way as that of the inside solutions, except that no gelatin was added to the outside solutions. The concentration of salt in each outside solution was made identical with the concentration of salt in the corresponding inside solution; also the pH was

[4] Field, A. M., *J. Am. Chem. Soc.*, 1921, xliii, 667.

identical in both cases. In order to eliminate CO_2 contamination, the outside solutions were poured into 500 cc. wide neck Pyrex Erlenmeyer flasks, provided with two-hole rubber stoppers. One hole served for the passage of the manometer tube while the other hole was fitted with a soda-lime tube. The top of the manometer tube was also closed by means of a soda-lime tube. The experiments were performed in a constant temperature water bath at 24°C. The maximum height of osmotic pressure was usually reached in about 6 hours, but the experiment was allowed to proceed for 11 hours in order to insure complete equilibrium. The given data were taken after 11 hours in each case.

The various salts whose depressing effect on the osmotic pressure of sodium gelatinate of pH 11.0 was studied consisted of the following groups.

(*a*) Sodium salts containing various univalent anions; namely, NaCl, NaBr, NaI, $NaNO_3$, NaCNS, Na acetate.

(*b*) Sodium salts containing anions of valencies higher than 1, such as Na_2SO_4, Na_2 tartrate, Na_2 oxalate, Na_3 citrate, $Na_4Fe(CN)_6$.

(*c*) Chloride salts containing univalent cations, such as NaCl, KCl, LiCl.

(*d*) Chloride salts containing divalent cations, such as $CaCl_2$ and $BaCl_2$.

Salts with cations of a valency higher than 2 are insoluble in an alkaline solution and naturally could not be used in this study.

The effects of the various salts on the osmotic pressure of Na gelatinate are plotted in Figs. 1 to 3. They show that all salts of a univalent cation exert the same depressing effect on the osmotic pressure of the gelatin for equivalent concentrations of the cations no matter what the chemical nature of these cations, as long as they are all of the same valency, and no matter what the nature or even valency of the anions.

Thus all the salts of Groups (*a*), (*b*), and (*c*) exert the same influence for equivalent concentrations of the cation.

But when the effect produced by the salts of these groups is compared with the effect produced by the salts of Group (*d*), such as $CaCl_2$ and $BaCl_2$, it is readily seen that these last salts depress the osmotic pressure of Na gelatinate to a considerably greater extent than all

other salts with univalent cations. These experiments prove without any doubt that only the cations, *i.e.* those ions which carry positive charges as opposed to the negative charges carried by the gelatin ions,

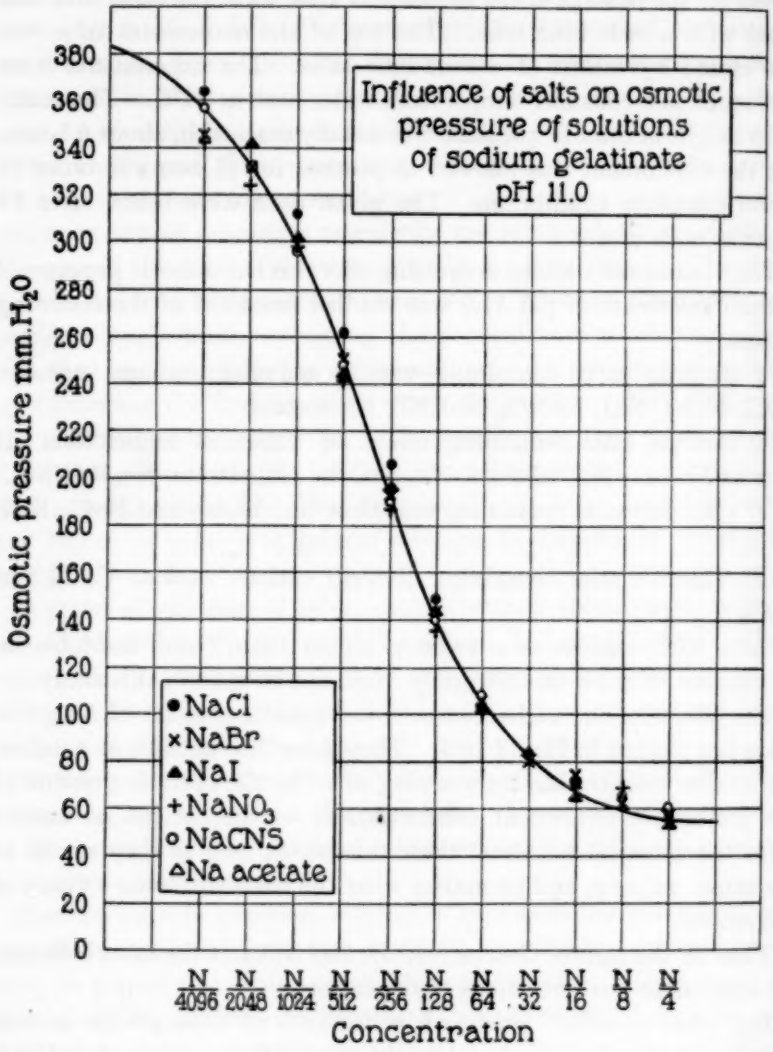

FIG. 1. Depressing effect of various sodium salts with monovalent anions on osmotic pressure of sodium gelatinate.

affect the osmotic pressure of the protein, and that for the same concentration of various salts the effect is greater the greater the number of charges carried by the cations, *i.e.* the greater the valency.

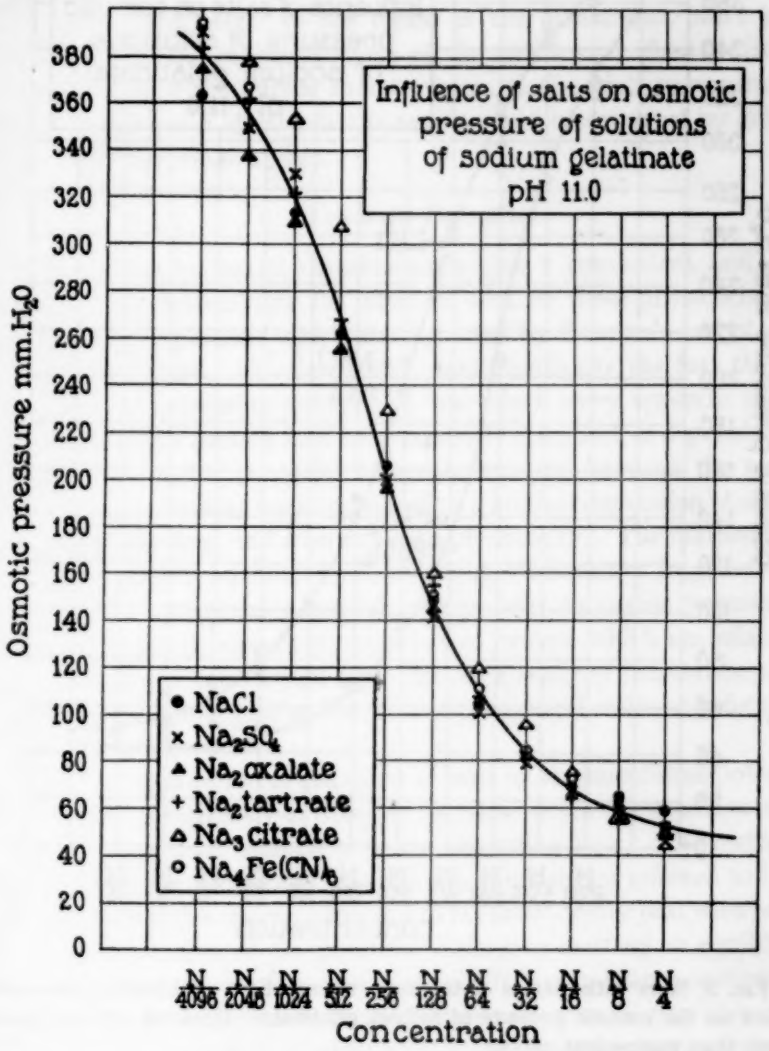

FIG. 2. All sodium salts, no matter what the valency or nature of their anions, have the same depressing effect on the osmotic pressure of sodium gelatinate.

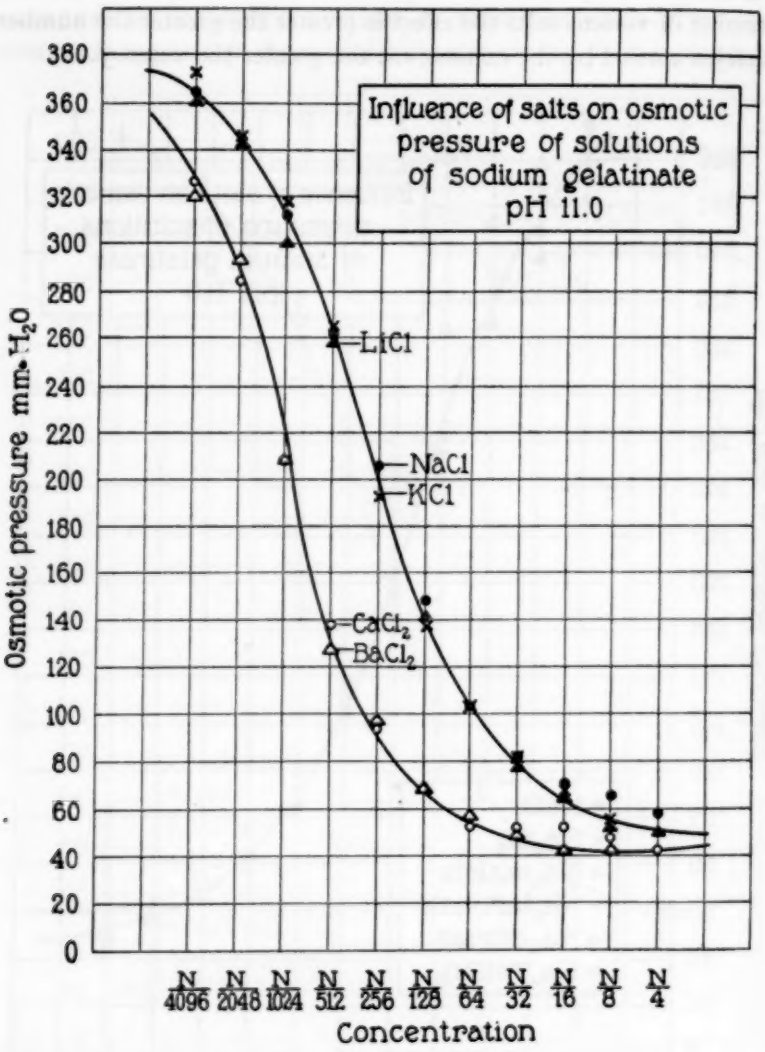

FIG. 3. Salts with cations of the same valency have an identical depressing effect on the osmotic pressure of sodium gelatinate. Divalent cations depress more than monovalent cations.

As the plotted curves show, the greatest rate of drop is in the lowest concentrations while in the higher concentrations the curves become asymptotic. Owing to technical difficulties it was almost impossible to take correct readings in the case of concentrations of salts higher than M/4, the height of the liquid in the manometer tubes being obscured by the rubber stoppers of the Erlenmeyer flasks. But as the flatness of the curves in the region of the high concentrations shows, not much change in the results could be expected by going a few concentrations higher.

Membrane Potentials.

Membrane potential measurements give a convenient and exact method for determining the effect of salts on those physicochemical properties of proteins which are regulated by Donnan's law of membrane equilibria. This law proves mathematically the fact that the addition of increasing amounts of ions which carry opposite charges to those of the protein ions have generally the effect of depressing the magnitude of the potential difference existing between the protein and the surrounding medium as well as a gradual depression of osmotic pressure, swelling, and a certain type of viscosity. The mathematical proof is developed fully by Loeb,[2] and Michaelis.[5] In general, the curves for membrane potentials run parallel to curves representing the other physicochemical properties of protein which are related to the membrane equilibrium law. Hence a study of membrane potentials by itself may give a clear idea of the general colloidal behavior of salts toward proteins.

The determination of the effect of salts on the membrane potentials of sodium gelatinate was done on the same series of experiments that had been used for osmotic pressure determinations. The method of measuring the membrane potentials is the same as referred to in the first paper of this series. Tables I to III show clearly that with respect to the depression of the potential difference existing at equilibrium between protein solutions inside of collodion membranes and protein-free salt solutions outside of the membranes there is no difference to be found in the action of various anions of various valencies as long as

[5] Michaelis,[1] p. 188.

the valency of the cations remains the same. Salts containing divalent cations such as Ca^{++} or Ba^{++} depress the potential differences much faster with increase in concentration of the added salt than

TABLE I.

Influence of Sodium Salts Containing Various Univalent Anions on Membrane Potentials of Sodium Gelatinate of pH 11.0.

The inside gelatin solution is in all cases negative towards the outside solution.

	Equivalent concentrations.											
	0	N/4096	N/2048	N/1024	N/512	N/256	N/128	N/64	N/32	N/16	N/8	N/4
	P.D. in millivolts.											
NaCl	21.0	21.0	19.5	17.5	13.5	10.0	5.5	4.0	2.0	1.5	0.5	0.5
NaBr	24.0	21.5	19.5	18.0	14.0	10.0	8.5	4.0	2.0	2.0	1.0	0
NaI	24.0	21.0	20.5	15.5	12.0	9.5	6.0	4.0	2.5	1.5	1.0	1.0
$NaNO_3$	23.0	20.0	18.5	14.5	11.0	9.0	5.5	3.0	1.5	0	0	0
NaCNS	23.5	20.5	18.5	16.0	12.0	9.0	5.5	3.5	2.2	1.3	1.0	1.3
Na acetate	22.5	20.5	18.5	14.5	13.5	8.5	4.5	3.5	2.5	1.3	1.0	0.5

TABLE II.

Influence of Sodium Salts Containing Anions of Various Valencies on Membrane Potentials of Sodium Gelatinate of pH 11.0.

	Equivalent concentrations.											
	0	N/4096	N/2048	N/1024	N/512	N/256	N/128	N/64	N/32	N/16	N/8	N/4
	P.D. in millivolts.											
NaCl	21.0	21.0	19.5	17.5	13.5	10.0	5.5	4.0	2.0	1.5	0.5	0.5
Na_2SO_4	21.5	21.5	19.5	16.5	11.5	9.0	5.0	2.5	2.0	1.0	0.5	0.5
Na_2 tartrate	22.5	20.5	18.5	16.5	12.5	8.5	5.0	3.0	1.8	1.0	1.0	1.0
Na_2 oxalate	23.0	22.0	19.5	15.0	13.0	8.5	5.5	3.0	1.8	1.0	0	1.0
Na_3 citrate	24.0	22.5	19.5	16.0	12.0	8.0	4.5	2.5	1.5	1.0	0.5	
$Na_4Fe(CN)_6$	22.5	22.0	20.0	16.5	10.0	8.5	4.5	2.0	1.5	1.0	0.5	0

salts with univalent cations. The results could be made even more striking by using sodium gelatinate of a lower pH than 11 where the potential difference between the gelatin and the outside solution is

considerably greater than at pH 11.0; but other difficulties would come in, such as the instability of the pH as referred to earlier. Another way of amplifying the results would be to use gelatin of high concentration, but then higher temperatures would have to be employed in order to prevent solidification. But even as the experiments for the P.D. measurement stand, the results are clear enough to dispel any doubt in the validity of the valency rule of the effect of ions on the mentioned property of proteins.

Effect of Salts on the Depression of Swelling of Sodium Gelatinate.

The general theory of swelling is discussed in detail in the first paper of this series. There it is also indicated that it was found advantageous to use powdered gelatin for swelling experiments instead of large blocks. In the case of blocks of gelatin it takes several days until equilibrium is established and in the meantime some of the gelatin dissolves or decomposes. The solubility effect is not related to the phenomena discussed in this paper and it varies with the kind of salt used. If powdered gelatin is used, the swelling equilibrium is established almost within 1 hour, and, if the experiments are conducted at a temperature not higher than 15°C., the solubility as well as hydrolysis can be reduced to a minimum. Another advantage of using powdered gelatin instead of blocks of gelatin is that whenever considerable solution of the gelatin does take place it will show itself immediately by the fact that the semidissolved gelatin granules will not permit the perfusion of the supernatant liquid through the mass of the gelatin, and the supernatant liquid cannot then be removed by filtration.

Gelatin particles of a definite size of grain, namely particles which went through a sieve with a mesh of 1/30 but not through a sieve with 1/60 of an inch, were brought to the isoelectric point and then without melting were kept in the refrigerator as a stock material for the following experiments. Equal amounts of 6.3 gm. each of the stock gelatin, each containing exactly 1 gm. dry weight of isoelectric gelatin were put into beakers and allowed to stand for about 18 hours in a moist chamber at 15°C. These were then added each to 150 cc. of various salt solutions of 15°C. All the salt solutions that were used were

made up in 0.01 N NaOH as it had been found by trial that 1 gm. of powdered isoelectric gelatin had at equilibrium a pH of about 11.0 when it was put into 150 cc. of 0.01 N NaOH. The pH of 0.01 N NaOH was found not to be affected materially by the addition of the various salts that were used.

It was thought desirable to change in these swelling experiments the system in which series of experiments were performed; namely, instead of working simultaneously with a series of solutions of various dilutions of one salt it was thought preferable to employ a series consisting of all the salts tried but all of an identical equivalent concentration.

TABLE III.

Influence of Salts Containing Various Cations on Membrane Potentials of Sodium Gelatinate of pH 11.0.

	Equivalent concentrations.											
	0	N/4096	N/2048	N/1024	N/512	N/256	N/128	N/64	N/32	N/16	N/8	N/4
	P.D. in millivolts.											
(*a*) Salts with univalent cations.												
NaCl	21.0	21.0	19.5	17.5	13.5	10.0	5.5	4.0	2.0	1.5	0.5	0.5
KCl	25.0	23.0	19.5	17.5	15.0	10.0	6.5	3.5	2.0	1.0	1.0	0.5
LiCl	24.0	22.5	20.0	18.0	14.5		8.0	4.0	2.0	1.0	0.5	0.5
(*b*) Salts with bivalent cations.												
$CaCl_2$	22.5	19.5	18.0	14.0	9.5	6.0	2.5	1.5	1.0	0	0	0
$BaCl_2$	24.5	21.5	16.0	14.5	10.5	6.5	4.0	2.0	0.5	0	0	0

Thus in Table IV, Experiment 1 consisted of solutions of an equivalent strength of N/2 of all the salts used, Experiment 2 consisted of N/4 of all the salts used, and so on. All the solutions of each series were used simultaneously and treated exactly alike. This was done in order to eliminate any errors due to slight changes in temperature, or differences in stirring, filtering, etc. A control solution consisting of N/100 NaOH without salt was used with each series. The gelatin was allowed to stand in the solution with frequent stirring for 2 hours at 15°C. The gelatin was then removed from the outside solution by filtration through cotton wool in weighed funnels. The liquid was

TABLE IV.

Influence of Salts on Swelling of Sodium Gelatinate of pH 11.0.

No. of experiment.	Equivalent concentration.	NaCl	NaBr	NaI	NaCNS	$NaNO_3$	Na acetate.	Na_2SO_4	Na_2 tartrate.	Na_2 oxalate.	Na_3 citrate.	$Na_4Fe(CN)_6$	KCl	LiCl	$CaCl_2$	$BaCl_2$	Control N/100 NaOH.
		cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.	cm.
	N/1							9.6	9.8		9.0				14.7	17.7*	
1	N/2	12.6	14.3	18.1*	16.2*	14.8	11.0	11.7	11.3		11.7	11.8	12.7	13.0	12.5	12.8	27.0
2	N/4	12.9	14.5	15.9	14.0	14.4	13.0	13.7	12.5	12.6	12.4	13.9	14.2	13.6	11.5	11.5	27.4
3	N/8	13.8	14.2	15.2	14.3	14.3	13.6	13.3	13.2	13.5	13.0	14.2	13.6	14.0	10.8	11.2	26.0
4	N/16	14.8	15.6	15.5	15.4	15.2	14.5	14.9	14.8	15.4	14.2	15.3	15.0	15.2	11.8	11.4	27.4
5	N/32	16.6	16.4	16.7	16.6	16.6	16.8	16.9	16.7	17.1	16.0	16.6	16.9	16.4	11.3	10.8	
6	N/64	19.6	19.6	19.6	18.8	19.5	19.4	19.5	19.2	19.9	18.7	19.6	19.0	18.9	12.2	12.5	26.8
7	N/128	21.1	21.1	21.6	20.6	21.2	21.0	20.9	21.4	21.6	22.3	21.7	21.8	21.8	14.7	15.2	27.4
8	N/256	24.4	24.2	24.5	23.6	23.7	24.5	23.8	24.1	24.8	24.7	24.6	24.9	24.5	17.9	18.9	28.3
9	N/512	24.5	24.9	25.0	25.2	25.4	24.7	25.3	25.2	25.6	26.0	26.1	25.4	25.8	22.9	23.8	25.7
10	N/1024	26.7	26.9	27.6	26.9	27.3	27.6	27.0	27.6	27.9		27.8	27.2	27.4	24.9	25.3	28.4
11	N/2048	27.0	27.2	27.8	27.9	28.7	28.8	27.6	28.2	28.5	28.7		28.1	27.2	26.2	26.6	28.5

* Incomplete filtration due to the semidissolved state of the gelatin.

allowed to drain off completely and the weight of gelatin was determined. Table IV gives the results of the various experiments. It is well to mention that the values for the weight of gelatin in the concentrations of salts higher than N/4 are a little too high due to the increase in the salt content of the gelatin. This amounts to an error in the highest concentrations used of about 1 gm., especially in the case of salts of high molecular weight such as NaBr, NaI, $BaCl_2$.

Clear cases of rapid solution of the gelatin were found in experiments with concentrations above N/8 of the following salts; namely, NaI, NaCNS, $BaCl_2$, in which the supernatant liquid could not be entirely removed by filtration. The weights obtained in these experiments indicate not only the weight of the gelatin but also the weight of a large portion of the supernatant liquid. Another irregularity was shown in the case of high concentrations of Na_3 citrate where a rapid drying and dehydration of the gelatin occurred similar to the action of alcohol on gelatin. But in concentrations lower than N/4 no cases of solubility were observed and no difficulty was experienced in removing the last drop of supernatant solution from the gelatin. Still, allowance has to be made for the liquid adhering to the enormous surface of the grains of gelatin and hence there is always a slight variation in the weight of the gelatin even in the control experiment. But this variation never exceeds a value of $\pm$ 1.5 gm. of the average value of 27.3 gm. for the control experiment. A detailed examination of the tabulated results as well as of the curves in Figs. 4 to 6 shows that the same rules that regulate the effect of ions on the osmotic pressure and the membrane potentials of sodium gelatinate hold also for the effect of ions on the typical colloidal property of gelatin; namely, on swelling. Thus, salts containing bivalent cations depress the swelling of the gelatin to a considerably greater extent than all other salts containing univalent cations. The anions appear to have no effect on the swelling no matter what their valency is. Cations of the same valency produced almost identical effects.

This holds true as long as we are dealing with the phenomenon of pure swelling. But as soon as other phenomena enter which do not depend on ionic activity, as for example solubility, then the rules derived from Donnan's law of membrane equilibria cannot be applied. This is what happens when salts in concentrations higher than N/4

are added to gelatin. There we are dealing not only with swelling of gelatin but also with cases of rapid solubility. Hence it is clear that

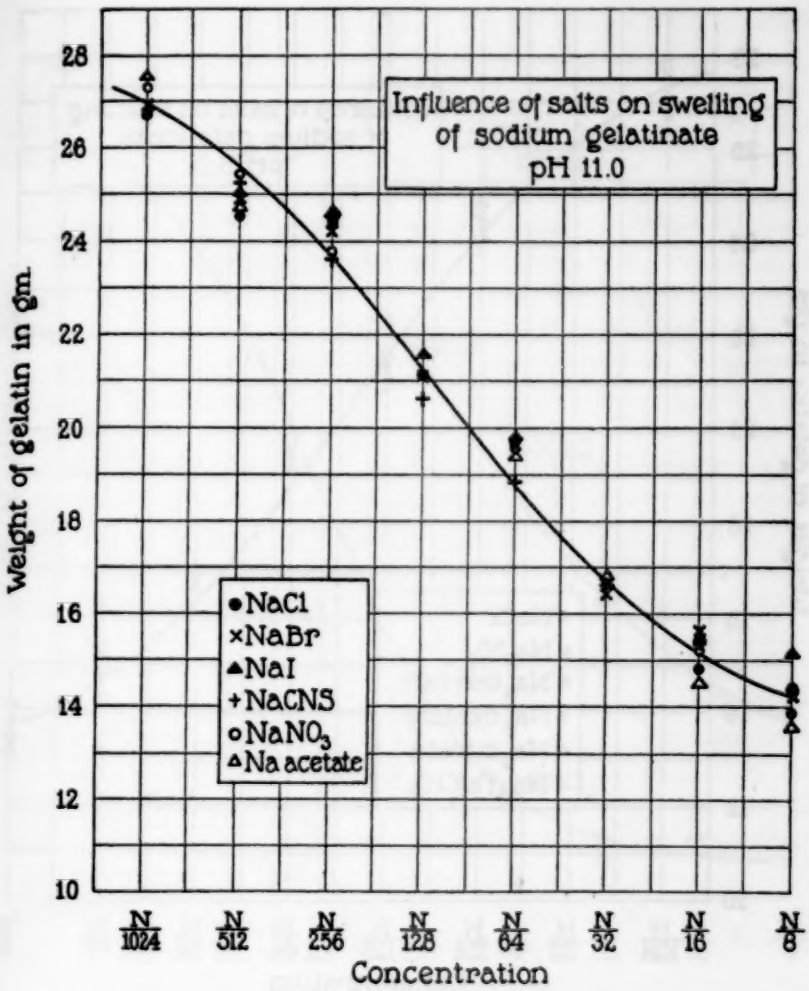

FIG. 4. Depressing effect of various sodium salts with monovalent anions on swelling of sodium gelatinate.

at the higher concentrations of salts the valency rule of ion effect cannot be applied as clearly as in the case of salt solutions of concen-

trations lower than N/4. This fact does not detract from the correctness of the valency rule for explaining the relation between salts and

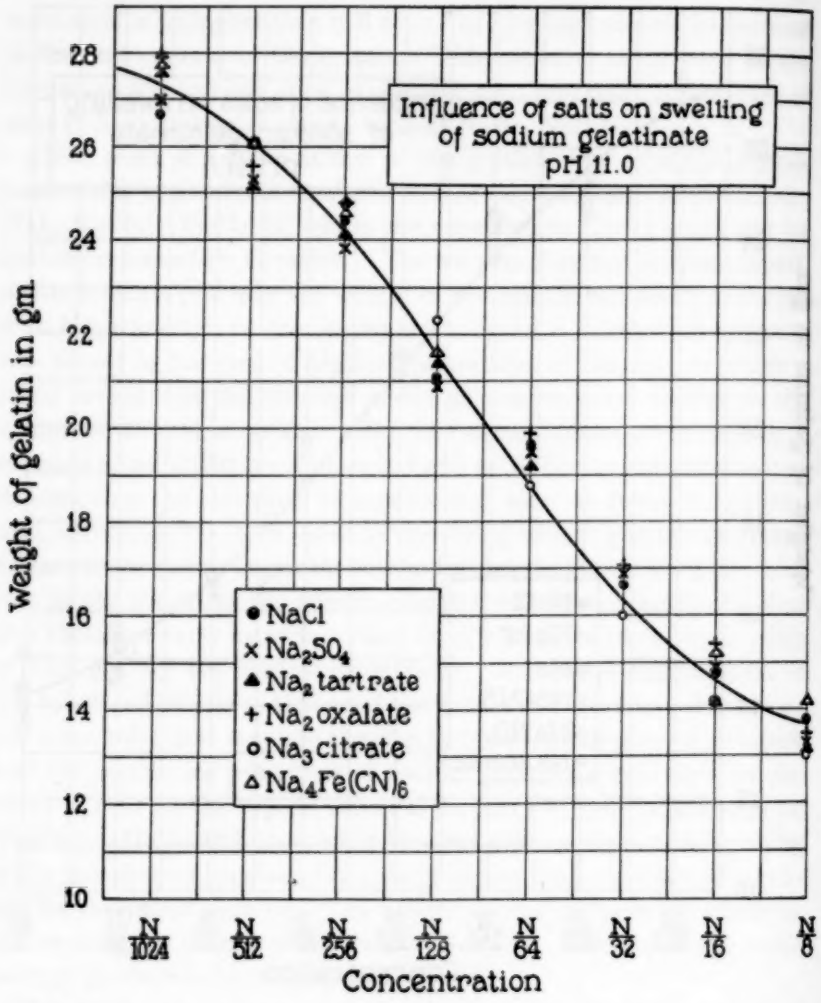

FIG. 5. The valency of the anions does not influence the depressing effect of sodium salts on the swelling of sodium gelatinate.

proteins, especially if it is remembered that very seldom physiological liquids are met with a salt content higher than N/4. As long as we

are dealing with the pure phenomenon of swelling, the valency rule can be applied as truly as in the case of osmotic pressure or membrane

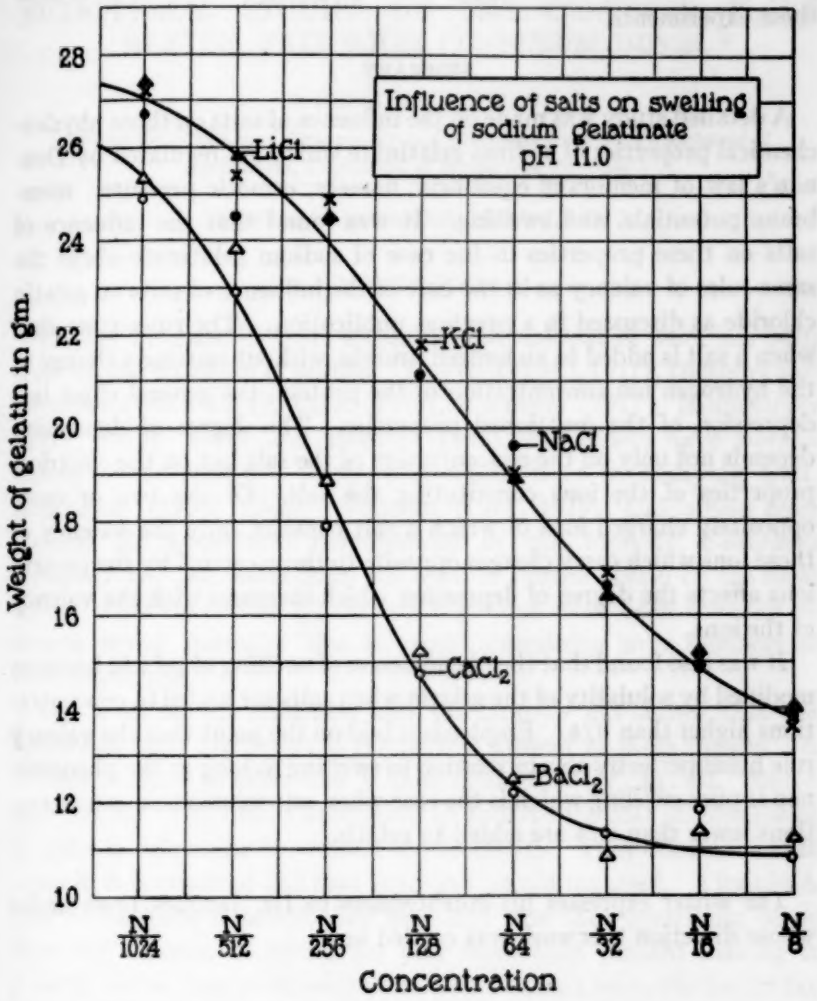

FIG. 6. The effect of the valency of the cations on the influence of salts on swelling of sodium gelatinate.

potentials. The claim of a number of authors that not only the valency but also the chemical nature of the various ions affect the swell-

ing of protein, and also the claim of other authors that some salts not only do not depress the swelling but even raise the swelling of some proteins certainly do not apply in the range of concentrations used in these experiments.

SUMMARY.

A detailed study was made on the influence of salts on those physicochemical properties of sodium gelatinate which are regulated by Donnan's law of membrane equilibria; namely, osmotic pressure, membrane potentials, and swelling. It was found that the influence of salts on these properties in the case of sodium gelatinate obeys the same rules of valency as in the case of the influence of salts on gelatin chloride as discussed in a previous publication. The rules state that when a salt is added to an ionized protein, without causing a change in the hydrogen ion concentration of the protein, the general effect is a depression of the mentioned properties. The degree of depression depends not only on the concentration of the salt but on the electrical properties of the ions constituting the salt. Of the two or more oppositely charged ions of which a salt consists, only the valency of those ions which carry charges opposite to those carried by the protein ions affects the degree of depression which increases with the valency of the ions.

It was also found that the phenomenon of swelling of gelatin becomes modified by solubility of the gelatin when salts are added in concentrations higher than N/4. Emphasis is laid on the point that the valency rule holds perfectly also in relation to swelling as long as the phenomenon is pure swelling which is the case when salt solutions of concentrations lower than N/4 are added to gelatin.

The writer expresses his indebtedness to Dr. Jacques Loeb under whose direction this work was carried out.

NUTRITIONAL STUDIES ON THE CONFUSED FLOUR BEETLE, TRIBOLIUM CONFUSUM DUVAL.*

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INTRODUCTION.

During the past 15 years a considerable amount of work has been done on the nutrition of insects. Guyénot (1913, 1917), Delcourt and Guyénot (1910, 1911), Loeb (1915), Northrop (1917), Baumberger (1917, 1919), Bacot and Harden (1922), and others have worked chiefly on the fruit-fly, *Drosophila*, which lives in decaying fruit. Bogdanow (1906, 1908), Wollman (1911, 1919), and others have studied the nutritional requirements of the blow-fly, *Calliphora vomitoria* L., but aside from the work of Portier (1919) on the meal worm, *Tenebrio molitor* L., the investigations have been confined to insects which normally live in media containing large numbers of microorganisms.

The work on the fruit-flies and the blow-fly has shown that the microorganisms are essential for normal growth in these insects. A further analysis of their diet is thus rendered very difficult. Bacot and Harden (1922) have attempted to analyze the diet of *Drosophila* in order to determine its vitamin requirements. They concluded that vitamin B is required and that vitamin C is not required. Vitamin A may be required although they were not sure that something other than the vitamin of butter fat may have been present making it possible for the flies to develop in the yeast extract when the butter fat was present but not in its absence. However, these authors make no mention of the fact that Baumberger (1919) was able to rear *Droso-*

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phila on yeast alone. Neither do they suggest that something other than the vitamin B in the yeast extract may furnish the necessary nutrients.

Portier (1919) got complete development of *Tenebrio molitor* in flour which had been sterilized at 130°C. This author is sure that certain granules found in the epithelium of the digestive tract are not symbionts, and that his experiments prove that *Tenebrio* can live in a sterile medium and that microorganisms are in no way essential to its normal growth. For this reason it has seemed that an insect, such as the confused flour beetle, *Tribolium confusum* Duval, which normally lives in flour or meal is a desirable form for a study in nutrition. The contents of the digestive tracts of several larvæ were stained and examined bacteriologically but no living organisms were found. In addition its normal life cycle requires but a few weeks, while that of *Tenebrio molitor* requires about 1 year.

In recording the rates of growth of insects, Baumberger (1919), Herms (1907), and others have used the weights of the larvæ. Hirschler (1914) reviewed various methods of measurement and adopted length and thickness. Neither length nor weight seem entirely satisfactory for two reasons: in the first place they do not lend themselves to accuracy and in the second place they take no account of the attainment of maturity. The length of nearly all insect larvæ varies greatly as the body elongates and contracts during locomotion. Consequently it is very difficult to assign a definite length to a larva at any time. The weighing of small larvæ is subject to the errors incident to the weighing of any small object and it also takes no account of variations in moisture content.

The attainment of maturity cannot be recorded by either the measurement or the weight for, as Herms (1907), Baumberger (1919), and many others have found, larvæ may reach maturity and pupate at various sizes. Herms found that he could control the size of the pupæ and adults within certain limits by varying the amount of food eaten by the larvæ of *Lucilia*. Those which fed longest grew largest and became the largest adults while those which fed the shortest length of time transformed while they were small Overfeeding was found to prolong larval life due to retarding pupation and in some cases they did not pupate at all. Popovici-Boznosanu (1910) obtained the

same results with *Osmia rufa* and concluded that maturity is reached at smaller size when food is reduced and at large size when food is plentiful.

Wodsedalek (1917) found that the museum beetle *Trogoderma tarsale* could endure starvation for a long time, molting its skin and decreasing in size until it again obtained food when it resumed growth.

From these results and the general knowledge that the adult insects of nearly every species vary in size it may be concluded that growth, in the sense of increasing size, may be distinguished from the attainment of maturity. Kunkel (1918) believes that the size of flesh-fly pupæ may be controlled by feeding ductless glands. It is later stated that mammalian thyroid retards growth and reduces size while thymus hastens pupation. But his data show such small differences of size and time that they would seem to be within the limits of experimental error. Dewitz (1902, 1913) believes that an oxidase controls the process of metamorphosis and that pyrogallic acid and CO_2 may retard it. Baumberger (1919) believes that certain changes in the nervous system and other organs must go on at a certain (minimum) rate if the minimum food substances are present. This might account for the variation in size when maturity is reached. However this may be, there would seem to be a good possibility of studying growth and the attainment of maturity in insects as affected by various foods.

The object of the present work was to determine whether growth and maturity could be controlled experimentally and whether the flour beetles could be used in nutritional work of broad biological application. The experiments which furnished the material for this paper consisted of two series. The first series is a study of individual insects while the second series is a study of the populations of certain cultures. The proteins used in the second series of experiments were furnished by Dr. R. A. Gortner who also contributed many valuable suggestions during the course of the work.

EXPERIMENTAL.

Experiments with Individual Insects.

In the first series of experiments the measurements of the head have been used as the criterion of growth (Mercer (1900) and Chapman

(1914–15)). Since the head capsule is chitinous and can expand only at the time of molting, the number of times which the larva has molted can readily be determined by measuring the width of the head. One has only to know the number of molts required for maturity in order to determine just what portion of the larval life has been completed at any particular time. The head measurement also serves to distinguish the undersized larvæ or "starvation forms" which may have more than the usual number of molts. When length or weight are relied upon it is impossible to know whether a larva of a certain size is about to pupate, is an undersized but mature larva, or whether it is still immature. When the head measurements are followed, the undersized mature larvæ can be distinguished from the immature larvæ by the greater number of molts which they have experienced. *The head measurement is thus a measurement of maturity or development as well as of growth*, when time is taken into consideration.

The larvæ of *Tribolium confusum* have been cultured in various foods including different grades of wheat flours and several of the so called wheat flour substitutes. It was found that the records in the wheat flours were very similar and therefore they were adopted as controls in all of the experiments. In plotting the results these controls were so plotted that the curve of development would bisect the angle of the ordinate and the abscissa. This was done by letting x, the growth between two molts, equal y, the number of days between these two molts. Maturity is thus measured by the number of molts and growth by time. The curve of development of the controls is a straight line at an angle of 45° representing the development of the larva from the time that it hatched from the egg until it molted and became a pupa.

In the period just before the last larval molt the larva not only grows as it has in the previous periods, but it also undergoes metamorphosis so that when it has molted once more it is no longer a larva but has the structure of a pupa. The plotting of the curve of development in such a way that it will be a straight line up to the time of the emergence of the pupa, assumes that the process of metamorphosis, which is undergone during the prepupal period, has a normal rate in proportion to the rate of growth which has preceded it.

A study of the curves for the five grades of wheat products shows that the lower grades retard growth very slightly. These products range from the middlings flour, the highest grade, through the sizings flour, low grade flour, and tailings flour, to the bran, which is the coarse

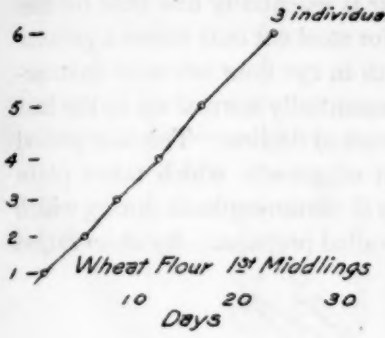

FIG. 1.

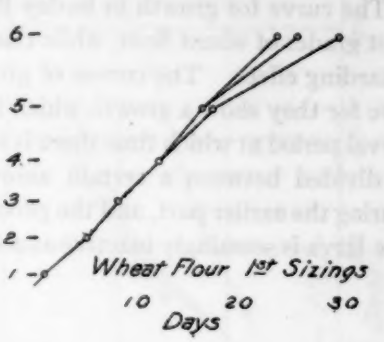

FIG. 2.

FIG. 1. Curve of larval development in wheat flour first middlings. Time plotted against the normal duration of instars.

FIG. 2. Curve of larval development in wheat flour first sizings.

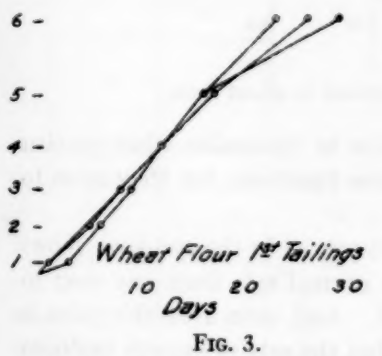

FIG. 3.

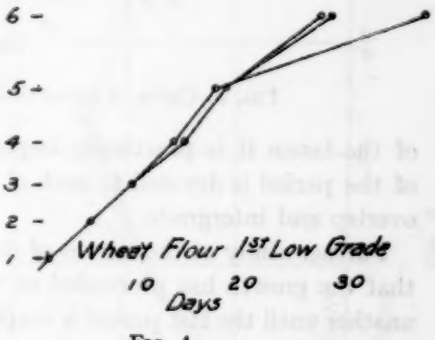

FIG. 4.

FIG. 3. Curve of larval development in wheat flour first tailings.

FIG. 4. Curve of larval development in wheat flour first low grade.

outer portion of the wheat kernel. Each individual curve on these graphs represents the record for an individual beetle and it will be noticed that there are some slight differences in the rates of development among individuals fed on the same food. In the middlings,

however, three individuals had exactly the same curve. The lower grades of the wheat flour not only retard growth to a slight degree, but they also retard metamorphosis, as is indicated by the break in the curve during the last larval period.

The curve for growth in barley flour is essentially like that for the best grades of wheat flour, while that for steel cut oats shows a greater retarding effect. The curves of growth in rye flour are most instructive for they show a growth which is essentially normal up to the last larval period at which time there is a marked decline. This last period is divided between a certain amount of growth which takes place during the earlier part, and the process of metamorphosis during which the larva is seemingly inactive as a so called prepupa. By observation

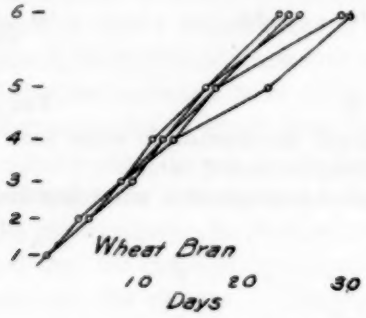

FIG. 5. Curve of larval development in wheat bran.

of the larvæ it is practically impossible to determine what portion of the period is devoted to each of these functions, for they seem to overlap and intergrade.

Further study of the curves of development in the rye flour shows that the growth has proceeded at the normal rate from one molt to another until the last period is reached. And, even after this point is reached there is no reason to believe that the rate of growth has been changed but rather that it is the new process of metamorphosis which is introduced at this time which is responsible for the change in the curve during this period alone. This seems to be substantiated by the fact that the size of the adults is very constant regardless of whether the larval period was short or long. Upon the basis of this assumption, which seems to be well founded, the deviation of the curve during the

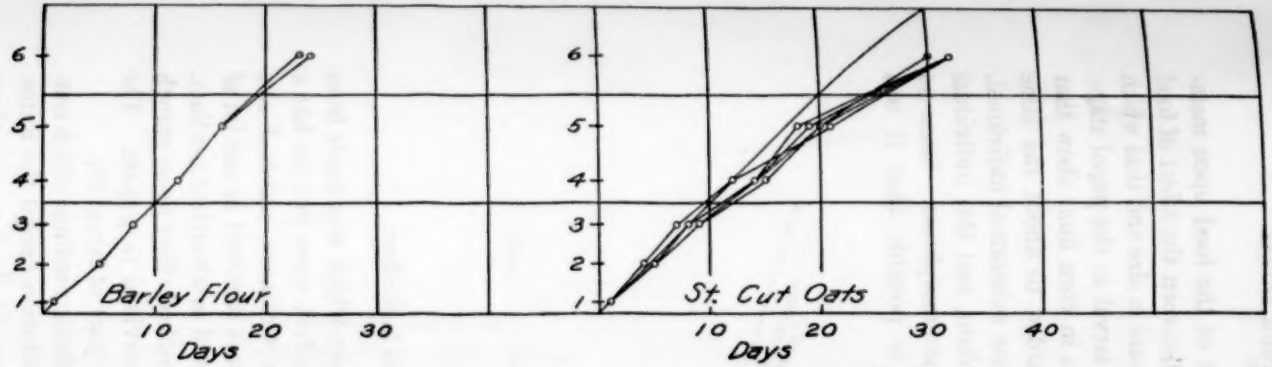

FIG. 6.

FIG. 7.

FIG. 6. Curve of larval development in barley flour.
FIG. 7. Curve of larval development in steel cut oats.

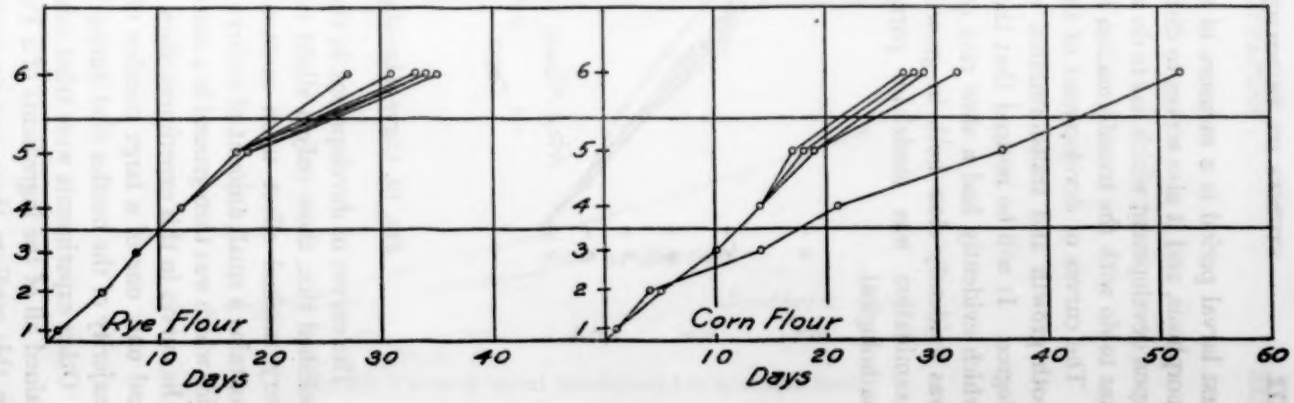

FIG. 8.

FIG. 9.

FIG. 8. Curve of larval development in rye flour.
FIG. 9. Curve of larval development in corn flour.

last larval period is a measure of the effect of the food upon metamorphosis, and it also serves to distinguish between the effect of food upon development which has to do with increase in size and that which has to do with the transformation from the larval to the pupal stage.

The curves of development of the beetles in corn flour show that both growth and transformation were retarded to about the same degree. It will be noticed that there was one subnormal individual, which evidently had a slow rate of metabolism, and this individual was evidently slow both in growth and metamorphosis. Since no examination was made for parasites it is possible that it was pathological.

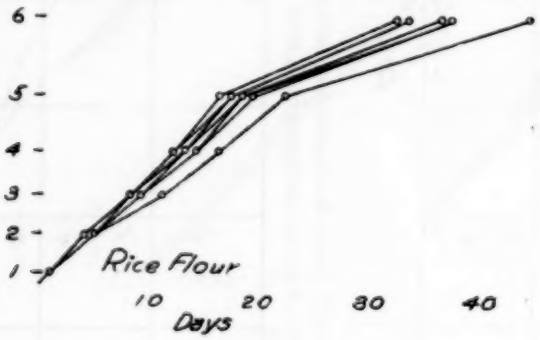

FIG. 10. Curve of larval development in rice flour.

The curves of development in the rice flour, which was made from polished rice, show only a slight retarding effect upon growth but a very marked effect upon metamorphosis. Ordinary polished rice contains a small amount of embryo, which was removed in one lot of rice which was then ground in a mortar and used as a nutrient medium. The curves in this experiment show a very marked effect upon growth and only one of a large number of larvæ survived to pupate. The majority of the beetles died during the early part of larval life.

Other experiments were tried using a synthetic medium which contained all of the ingredients of a Pasteur's solution except the water. In this medium there was no protein. None of the larvæ in these experiments survived to pupate and the mortality was very high

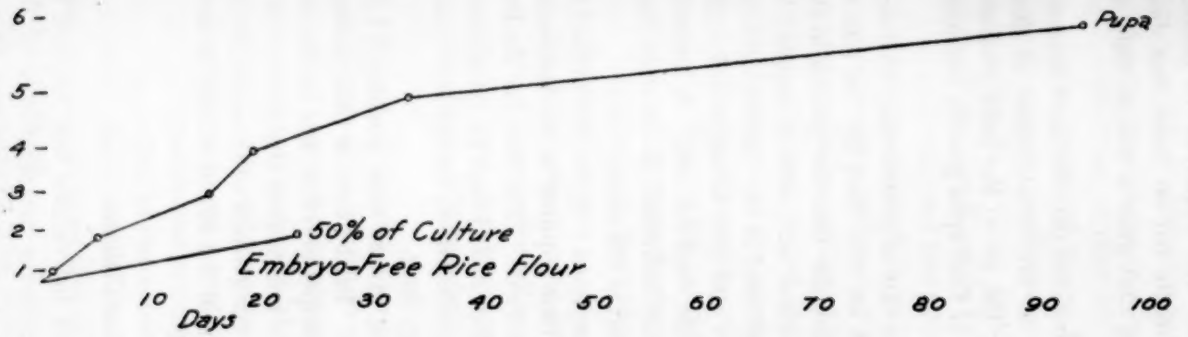

FIG. 11. Curve of larval development in embryo-free rice flour.

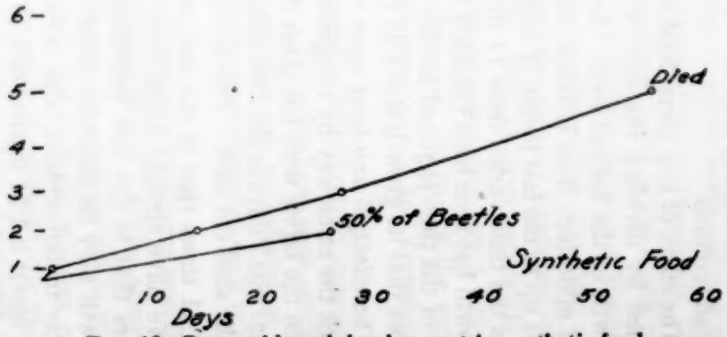

FIG. 12. Curve of larval development in synthetic food.

during the early periods. No method was employed to prevent cannibalism and the survivors undoubtedly fed on those that died. Nevertheless it seems rather surprising that growth was as rapid as the curves show it to have been.

The results as shown in these curves indicate that the head measurement is a very satisfactory criterion of the development of these insects. Furthermore it is possible by the use of the head measurement to distinguish between the effect of food upon growth and upon metamorphosis.

The effect of any given food upon the rate of growth may be determined by dividing the rate of growth on this food by the rate of growth in the normal food. In this case the time for growth in the first middlings flour is taken as the normal and, since it required 16 days up to the last instar, it may be expressed as 16. In the steel cut oats the time varied from 17 to 20 days and may be expressed as 20. Growth has then been retarded 1.25 in the steel cut oats. A comparison of the time required for the abnormal individual in the corn flour shows that its growth was 2.25 times that in the normal diet.

The effect of any food upon metamorphosis may be determined in much the same way by comparing the time required in the last instar when the larvæ were fed upon different foods. The time for the last instar was 7 days in the first middlings flour and 11 days for the average in the steel cut oats. This gives us a prolongation, for the last instar, of 1.51 times that in the first middlings flour.

We have already found that the first instars were prolonged 1.25 times that in the first middlings flour. In the case of these instars growth may be assumed to be the principal activity, but in the last instar metamorphosis also occurs. We notice that the ratio in this last instar is 1.51 instead of 1.25. The comparison of these two ratios may therefore represent the comparison of the effects of this food on growth alone and upon metamorphosis.

Experiments with Mass Culture.

The study of mass culture instead of individuals was resorted to because the task of making the micrometer measurements of the individuals was so exacting as to make it impossible for more than a few insects to be under observation at one time. The mass culture

method was chosen with the hope that it might develop into an easy routine which would facilitate nutritional investigations. Since these beetles are small, large numbers of them can be kept in a small space and the shortness of their life cycle makes many generations possible in a short period of time. Consequently they would be valuable animals to use in nutritional work if they proved to be sufficiently like higher animals in their requirements. (See Osborne and Mendel (1913, 1914); also Kianizin (1915–16).)

The death rate and times of emergence of adults in the cultures were used as the measure of the nutritional value of the various foods. The death rates were plotted to give a summation curve of the death rates of each population much as Davey (1919) did for cultures of the adult beetles. The per cent of the total population which was dead each day was recorded on the larval curve and the per cent which had transformed was recorded on the adult curve. When a larva pupated it was counted as dead on the curve for the larval population but it was also recorded on the curve for transformation. At the end of the experiment the per cent dead plus the per cent which had transformed always equaled 100.

Each culture was started from eggs which were carefully guarded to prevent any infestation with parasites. The containers were sterilized and larvæ were examined from time to time but no infestation was found. About 1,000 newly hatched larvæ were placed in each culture at the start.

The control cultures were in wheat flour and all cultures were kept side by side in an incubator maintained at 28°C. Lacking adequate humidity control it was not possible to get the same results in winter when the humidity in the laboratory was low as in the summer when it was high. In order to continue the work throughout the year it was necessary to run a control culture in wheat flour along with each series of experimental cultures. A comparison of the control curves for summer and winter shows that in the latter metamorphosis was retarded. On the other hand, control cultures which were run in duplicate at the same time of the year gave curves which were practically identical.

The populations were counted each day to record the number of living larvæ. There can be little doubt that the daily handling of the

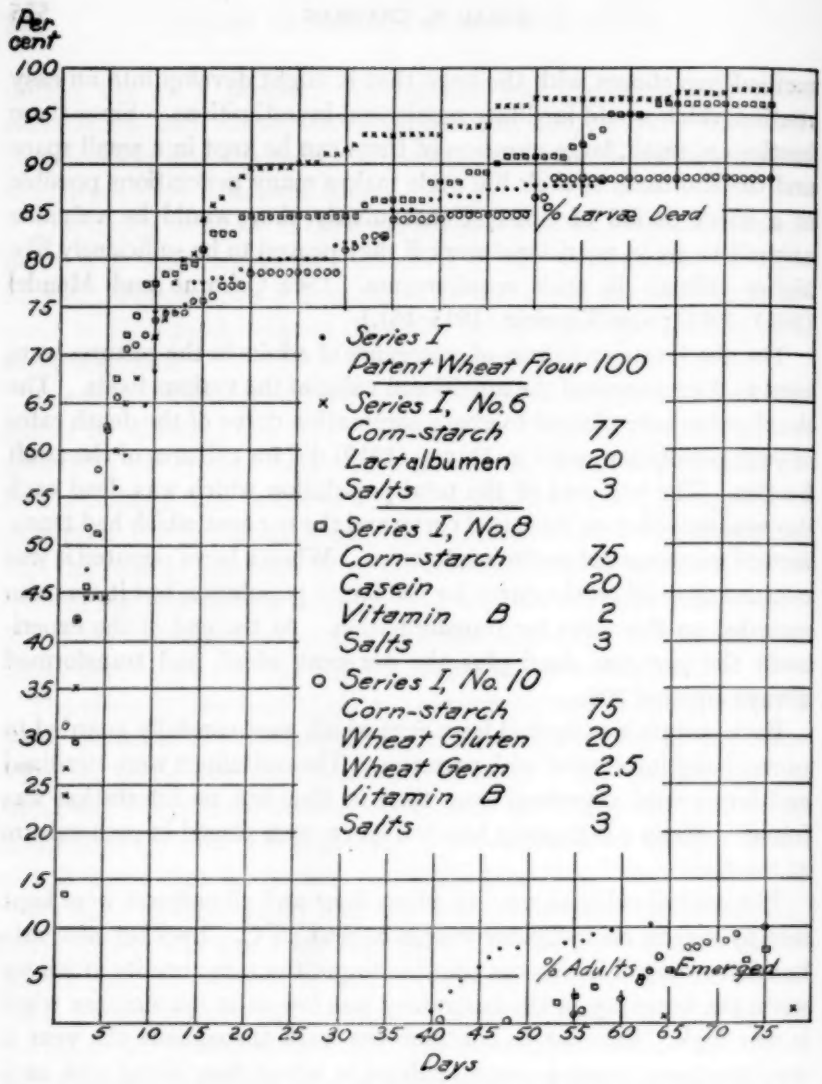

FIG. 13. Curve showing larval death rate expressed as the per cent of larvæ dead each day and the per cent of adult beetles emerged from a culture in wheat flour at high humidity.

Larval death rate in corn-starch 77 gm., lactalbumen 20 gm., and Osborne and Mendel salt mixture 3 gm. (Emergence of adults in lower right.)

Larval death rate in corn-starch 75 gm., casein 20 gm., vitamin B 2 gm., and salts 3 gm.

Larval death rate in corn-starch 75 gm., wheat gluten 20 gm., wheat germ 2.5 gm., vitamin B 2 gm., and salts 3 gm. (Adults emerged in lower right.)

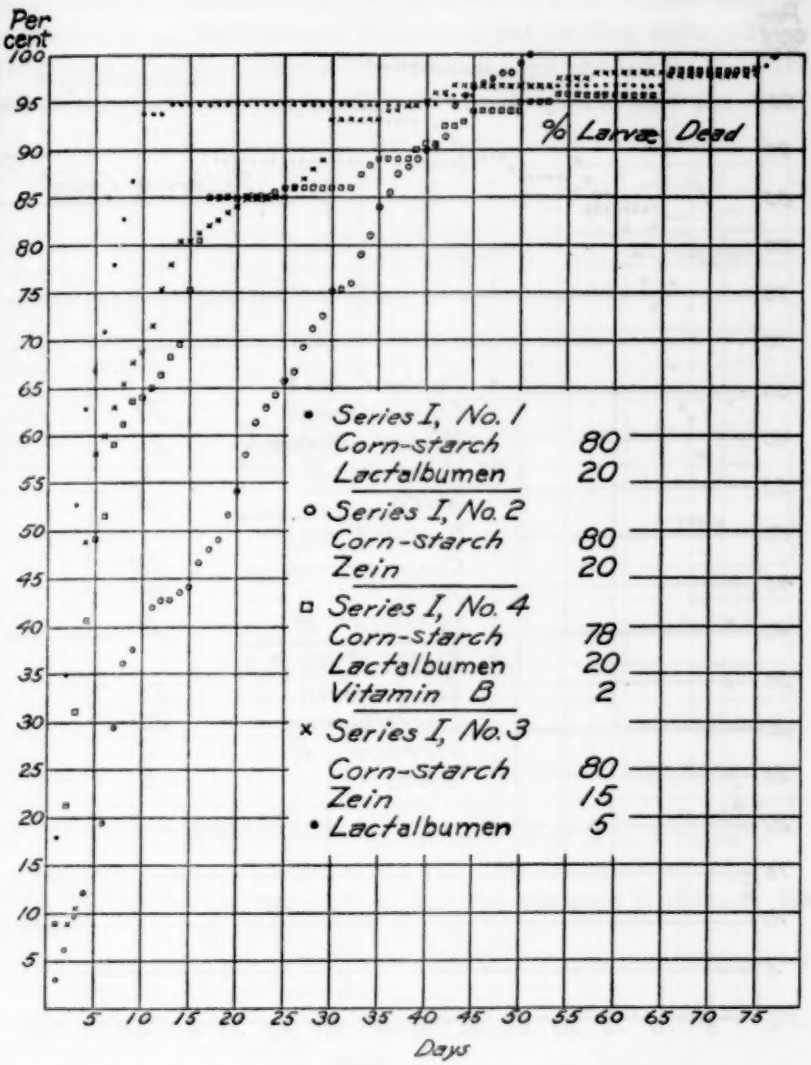

FIG. 14. Larval death rate in corn-starch 80 gm. and lactalbumen 20 gm.
Larval death rate in corn-starch 80 gm. and zein 20 gm.
" " " " " 78 " lactalbumen 20 gm., and vitamin B from wheat germ or dextrin, 2 gm.
Larval death rate in corn-starch, 80 gm., zein 15 gm., and lactalbumen 5 gm.

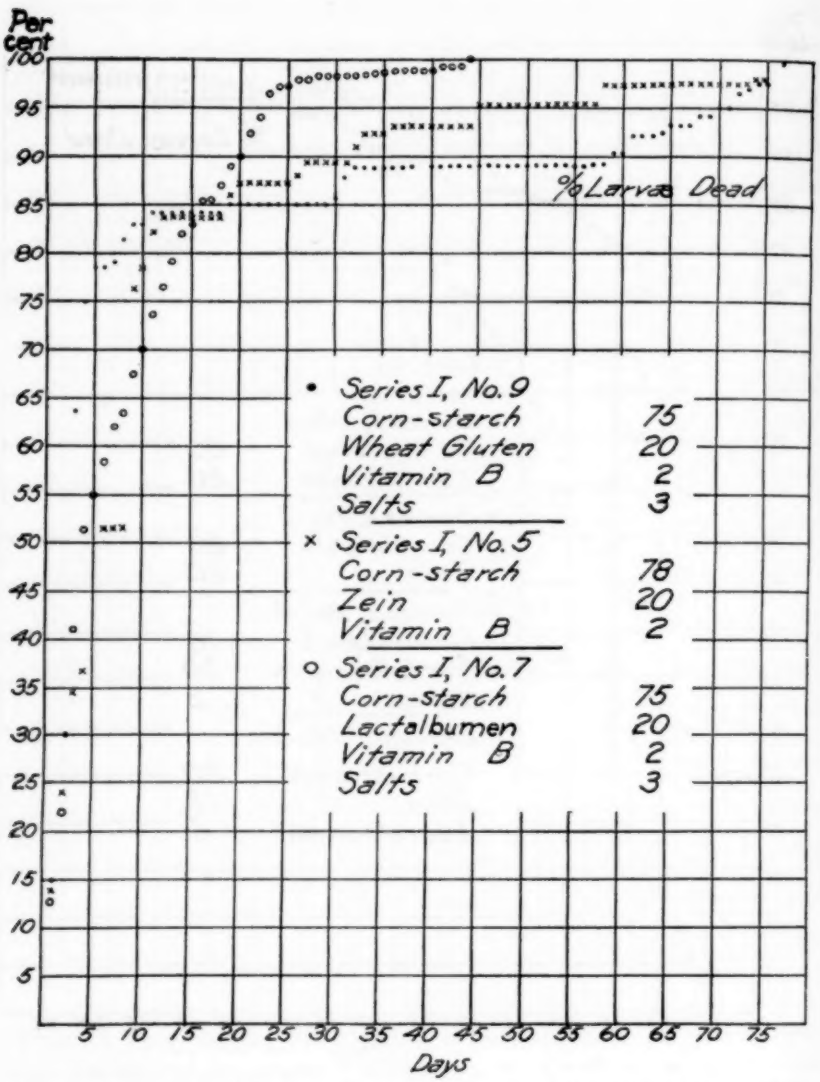

FIG. 15. Larval death rate in corn-starch 75 gm., wheat gluten 20 gm., vitamin B 2 gm., and salts 3 gm.

Larval death rate in corn-starch 78 gm., zein 20 gm., and vitamin B 2 gm.

" " " " " 75 " lactalbumen 20 gm., vitamin B 2 gm., and salts 3 gm.

larvæ increased the death rate, as was shown by a comparison with the number of pupæ from cultures which were not handled daily. However, since all of the cultures were handled alike the results from the different cultures are comparable. It was also found that the deatl

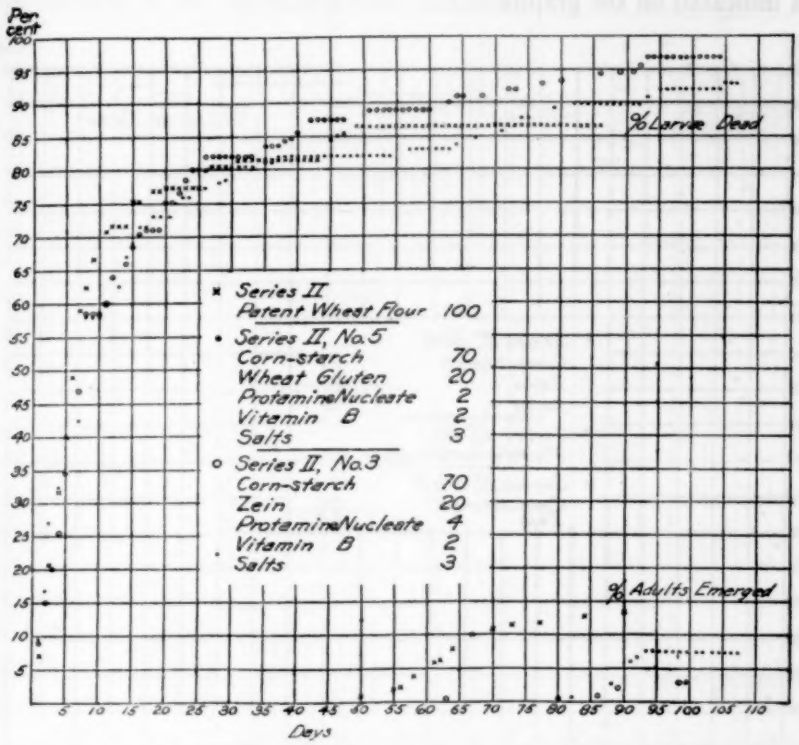

FIG. 16. Larval death rate in wheat flour also emergence of adults, at low humidity.

Larval death rate in corn-starch 70 gm., wheat gluten 20 gm., protamine nucleate 2 gm., vitamin B 2 gm., and salts 3 gm.

Larval death rate in corn-starch 70 gm., zein 20 gm., protamine nucleate 4 gm., vitamin B 2 gm., and salts 3 gm.

rate was the same whether larvæ were kept separately or in mass cultures.

All of the foods used had Kingsford's corn-starch as the base to which various proteins, salts, and vitamin were added. The experiments follow the general plan of those of Osborne and Mendel (1913)

with mammals in order that the insects might be compared with other animals. The salt mixture of Osborne and Mendel (Loeb (1915)), lactalbumen, casein, zein, wheat gluten, protamine nucleate, vitamin from wheat germ, and the wheat germ itself were added to the starch as indicated on the graphs.

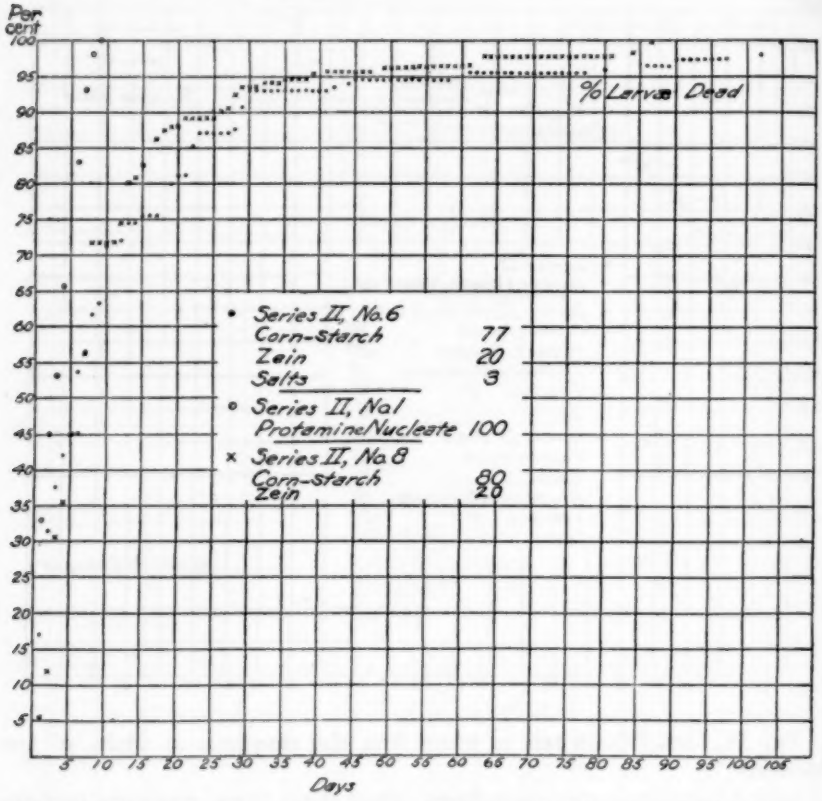

FIG. 17. Larval death rate in corn-starch 77 gm., zein 20 gm., and salts 3 gm.
Larval death rate in protamine nucleate 100 gm.
" " " " corn-starch 80 gm. and zein 20 gm.

The curves resulting from these experiments are not smooth but are marked by more or less abrupt breaks. These occur in about the same portions of all of the curves and at about the right intervals to represent the time of molting. Since molting is known to be a crisis in the lives of larvæ it seems reasonable to believe that death is more apt

to occur at this time than at any other. Observations on the individual insects support this view and a comparison of the control curves for summer and winter (high and low humidity) shows that these breaks are more abrupt in cultures exposed to dry conditions in which the molting of the dry skin is most difficult.

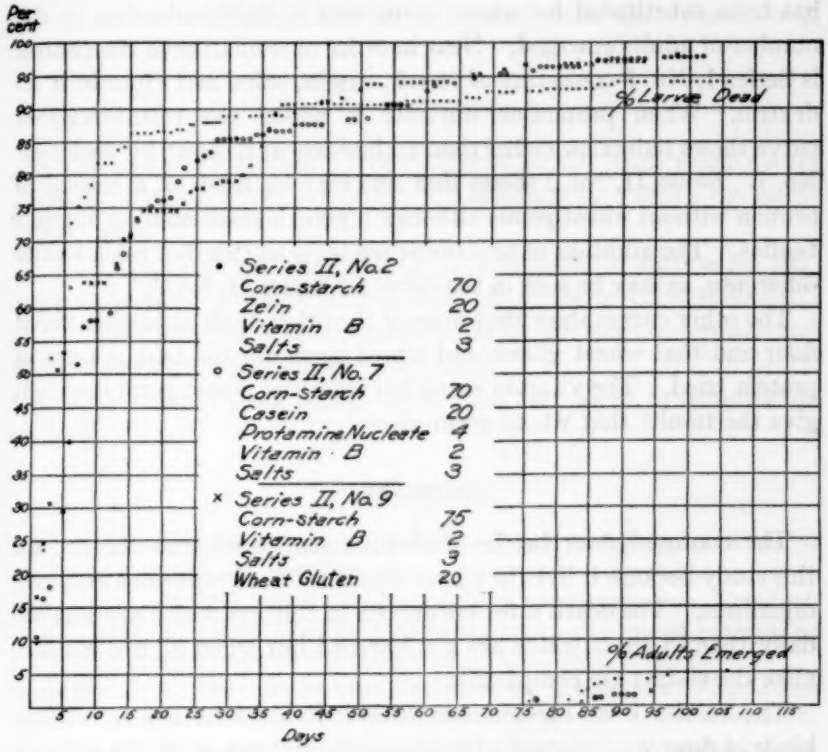

FIG. 18. Larval death rate in corn-starch 70 gm., zein 20 gm., vitamin B 2 gm., and salts 3 gm.

Larval death rate in corn-starch 70 gm., casein 20 gm., protamine nucleate 4 gm., vitamin B 2 gm., and salts 3 gm. (Adult emergence in lower right.)

Larval death rate in corn-starch 75 gm., vitamin B 2 gm., salts 3 gm., and wheat gluten 20 gm. (Adult emergence in lower right.)

Diet Series I, No. 10 containing starch, wheat gluten, wheat germ, vitamin B on dextrin, and salts gave almost exactly the same curve as the control in wheat flour, except that the transformation was delayed about 2 weeks. Diet Series I, No. 9 was identical with No. 10

except that it lacked the wheat germ. The death curve for this culture is like No. 10 up to about the time for transformation when the larvæ all died.

The next approach to the control curve is that for Series II, No. 5 which is the same as Series I, No. 10 except that protamine nucleate has been substituted for wheat germ with a slight reduction in the number of adults emerged. Next in order of similarity to the control is Series I, No. 8 consisting of starch, casein, salts, and vitamin B on dextrin. When protamine nucleate is added the transformation curve shows reduction rather than an increase as is shown by Series II, No. 7. Series II, No. 2 shows that zein may be used as a source of protein without wheat germs although it gives a small number of adult beetles. The addition of protamine nucleate to this diet makes little difference, as may be seen in the curve for Series II, No. 3.

The other curves show that none of the other foods approach wheat flour and that wheat gluten and wheat germ are the best sources of protein tried. The vitamin extracted from the wheat germ does not give the results that wheat germ gives.

SUMMARY.

The confused flour beetle (*Tribolium confusum*) was chosen for this study because it lives in a food which ordinarily contains no living organisms. The death rates are greater in cultures which are handled daily than in those which are not handled but when all are handled alike the results are comparable.

The results from experiments with individual beetles in various kinds of flour were plotted with instars (larval stages) on the ordinate and time in days on the abscissa, using the results from control experiments in wheat flour to determine the length of the various instars from an "$x = y$" formula. The curves of development were found to be straight lines throughout all but the last instar. The curve for the last instar during which the larva transformed deviated from the straight line in certain foods, notably rice flour.

When mass cultures were used the death and transformation curves were plotted for each synthetic food. A comparison of the curves from wheat flour and the synthetic foods shows that the first parts

of the curves are very much alike in all cases and that a few resemble the control in every respect except that the transformation curve has been moved back for a considerable time.

The death curves for the mass cultures are not smooth but show sudden increase in death at approximately the times of molting. These curves may therefore be compared with the records from individual beetles.

CONCLUSIONS.

1. Insects which normally feed in flour or similar foods which do not normally contain living organisms are satisfactory animals for nutritional work.

2. The requirements for growth, in the sense of increasing size, seem to be much less exacting than for maturity or transformation.

3. Wheat germ more nearly satisfies the requirement for growth and transformation than anything else tried.

4. The vitamin from wheat germ does not seem to supplement deficient diets.

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CHANGES IN THE STABILITY AND POTENTIAL OF CELL SUSPENSIONS.

II. The Potential of Erythrocytes.

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In the first paper of this series (Eggerth (1923–24)) it was shown that washed suspensions of *Bacterium coli*, when suspended in buffer mixtures commonly used for acid agglutination, underwent progressive changes, both in their potential and their stability. These changes were shown to be due to two factors: the extraction from the bacteria of a soluble protein which, by again combining with them, depressed their negative charge; and a further irreversible change of the cell or its membrane which also diminished the negative charge. In the experiments here described, a similar study was made of the changes in the potential of washed erythrocytes of the rabbit, sheep, and man.

The erythrocytes were always obtained from fresh, defibrinated blood; all experiments were completed on the same day that the blood was drawn. The cells were washed four times from a large volume of 0.85 per cent NaCl solution. In some experiments, they were washed further with 9 per cent saccharose solution. A 25 per cent suspension of the packed erythrocytes was then made in salt or saccharose solution, and one or two drops of this heavy suspension were added to 50 cc. of the solution in which they were cataphoresed. If the pH is more acid than 5.4, it is necessary to greatly dilute the erythrocyte suspension with 9 per cent saccharose solution before bringing to the required pH, otherwise clumping will occur. Concentrated red cell suspensions, dropped into acid solutions of low salt concentration, agglutinate immediately into sticky masses that cannot be broken up satisfactorily; by proper dilution of the suspension before mixing with the acid solutions, the erythrocytes remain unagglutinated for a

long time, no matter what the pH or the salt concentration. This indicates that the distance between the cells in the suspension is an important factor in determining their stability.

Many of the experiments were conducted with the erythrocytes in buffer mixtures. Phosphate mixtures were used for the range from pH 5.8 to 8.0; for the more acid ranges, the phthalate buffers of Clark and Lubs (1917) were used. The phthalate solutions were selected because they were found to be less hemolytic in the acid ranges than the acetate or lactate solutions used in the previous study. The anion in the phthalate buffer series is monovalent at the acid end, and divalent at the alkaline end of the series; hence the ion effect of the salt is not uniform at different H ion concentrations. However, when only the changes in the potential of the erythrocytes at a given pH are studied, this does not cause error.

The phosphate and phthalate solutions were made up according to Clark and Lubs (1917), except that KOH instead of NaOH was used, so as to have only one cation present. In order to have a uniform equivalent salt concentration (which the specifications of Clark and Lubs would not attain) the required amounts of 0.2 N alkali were added to the 0.2 N acid salts; this gives in each case a solution which is 0.2 N with respect to both the primary and the secondary salts present. For use, the 0.2 N buffers were diluted to the required concentration with 9 per cent saccharose solution.

Cataphoresis was done by the microscopic method, using a cell similar to the one described by Northrop (1921–22). The depth of the cell was 1 mm., and the fall in potential of the current was 5 volts per cm. In cataphoresing erythrocytes by this method, it is necessary to make measurements at different levels of the entire cell depth. In these experiments, the velocity was determined at the middle of each sixth of the cell depth. On filling a clean cataphoresis cell with a suspension of electronegative erythrocytes, one frequently finds that their rate of movement is distinctly slower in the upper than in the lower half of the cell. (See Column 1, Table I.) After a short period of use, especially in the more acid reactions, the rate of movement towards the anode becomes more rapid along the top (Columns 2, 3, and 4, Table I); in fact, at certain reactions, the most rapidly moving erythrocytes in the apparatus will be found in the topmost

layers. A concrete example of this phenomenon is shown in Table I which gives the observed times in seconds required for the erythrocytes to travel 100 micra, at the middle of each sixth of the cell depth.

In the first experiment shown in Table I the bottom of the cell was already covered with a film of erythrocytes, while the top was still clean glass. The potential difference between the clean glass top and the fluid was relatively large, and caused a considerable streaming to the cathode, this in turn slowing the movement of the red cells to the anode. The potential difference between the erythrocyte film on the bottom, and the fluid, was smaller in magnitude, though the

TABLE I.

The Velocity of Human Erythrocytes at Different Depths of the Microcataphoresis Cell.

Cell depth.	pH 5.6 (1)	pH 4.8 (2)	pH 4.0 (3)	pH 3.2 (4)
	sec.	*sec.*	*sec.*	*sec.*
1	−14.0	−12.0	−15.5	− 24.6
2	− 9.9	−10.9	−17.5	−100.0
3	− 8.0	−10.6	−23.4	+60.0
4	− 7.1	−10.2	−27.0	+37.0
5	− 8.0	−10.3	−25.2	+45.0
6	− 9.8	−10.7	−22.0	+70.0

Cataphoresis was done in 0.01 N phthalate buffers containing 9 per cent saccharose. Observations were made in the order given; the total time in which they were made was 22 minutes. The negative sign indicates movement towards the anode; the positive, movement towards the cathode.

same in sign, causing a lesser movement of the fluid to the cathode. At pH 4.0 and 3.2 the potential of the film of erythrocytes on the bottom is obviously near zero (as the velocity in the sixth or lowest depth is nearly that of the average velocity for all depths, showing absence of movement of the fluid). This is to be expected, as human erythrocytes in 0.01 N phthalate are isoelectric at about pH 3.5; and Northrop (1921–22) showed that a cell wall coated with bacteria reverses its charge under the same conditions as does the suspension. At these reactions (Columns 3 and 4, Table I), the migration of fluid along the top wall of the cell is toward the anode; it follows that the glass must be coated with something that is electropositive at pH 4.0

and 3.2. On dimming the illumination, the top of the cell is seen to be plastered with pale shadow forms, the stroma of hemolyzed erythrocytes. To these we must ascribe the electrical properties of the top wall of the cell.

When 0.01 N phthalate buffers were used, the observed velocity of human, sheep, and rabbit erythrocytes in the upper sixth of the cataphoresis cell, was equal to the average velocity of all depths at about pH 4.6. This means that the film of stroma was isopotential with the fluid at that pH, whereas intact red cells in this 0.01 N buffer series were isopotential at pH 3.5 (human); sheep cells were isopotential at pH 3.2, and rabbit cells at pH 4.0. In the presence of salts having monovalent ions, the stroma film was found to be isopotential at pH 4.7, as were also the intact erythrocytes of all three species.

Daily thorough cleaning of the cataphoresis cell is desirable. Rinsing with 5 per cent acetic acid cleans very well; alkali is not desirable, as it softens the de Khotinsky cement commonly used in constructing the apparatus. A piece of insulated copper wire is excellent for mechanical cleansing.

In Fig. 1 is shown the change in rate of movement of human erythrocytes kept in 0.01 N phthalate and phosphate buffer solutions made up in 9 per cent saccharose solution. The temperature was room temperature, usually about 18°C. The rates of movement are given as micra per second with a fall in potential of 1 volt per cm. Following the suggestion of Winslow, Falk, and Caulfield (1923–24), no attempt was made at actual calculation of the potential difference between cells and fluid; the high concentration of saccharose must seriously affect several of the factors in the Helmholtz-Lamb equation. It may safely be assumed that the potential of the erythrocyte varies as its rate of movement.

Fig. 1 shows that when human red blood cells are added to solutions at pH 5.2 or more acid, they become progressively less electronegative (or more electropositive). This change, though slow at pH 5.2, is more rapid in the more acid solutions, and is usually nearly complete in 2 hours. At pH 3.2 and 3.6, hemolysis of all the cells usually occurred at the end of that time. Some hemolysis likewise occurs at pH 4.0, though many cells still remain. At pH 4.4, hemolysis is very slight, even after 6 hours.

It will be observed that there is a slight diminution in the velocity of the erythrocytes at pH 5.6 and 6.6. This slight diminution also occurs at all reactions up to pH 9.2. It occurs whenever cells remain for some time in a solution of low salt concentration; erythrocytes that have been washed in saccharose solution, or have stood for some time in saccharose solution free from salt, quite regularly have a lower charge when placed in buffer solutions than those taken out of 0.85 per cent NaCl.

Changes in potential, similar to those shown in Fig. 1, were also observed when sheep erythrocytes were studied. Rabbit erythro-

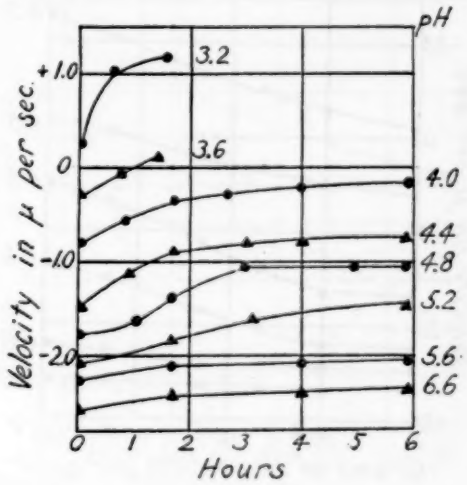

FIG. 1. Changes in the velocity of human erythrocytes in 0.01 N phthalate and phosphate buffer mixtures. Temperature 18°C.

cytes, on the contrary, show very little change in rate of movement when treated in this way; the curves are nearly flat. The entire reason for this difference between the behavior of rabbit red cells and the other two species studied, will require further experiments to clear up; though it seems in part accounted for by the fact that the rabbit erythrocytes are unusually resistant to the hemolytic action of the acid buffer mixtures used.

In determining the cause of the change in potential of *Bacterium coli* when this organism was incubated at reactions near pH 3.0, it was found that a soluble protein was extracted from the bacteria; this

protein, when added to a suspension of fresh bacteria, markedly reduced their charge. This suggests that the hemoglobin derived from those cells that have been hemolyzed, might be a cause of the changes in potential of the erythrocytes, as shown in Fig. 1. Experiment verified this supposition.

In the experiment shown in Fig. 2 human erythrocytes were hemolyzed with distilled water, and the stroma removed by centrifugation. The hemoglobin was considered as being 32 per cent of the weight of the packed erythrocytes. Various dilutions of hemoglobin solution

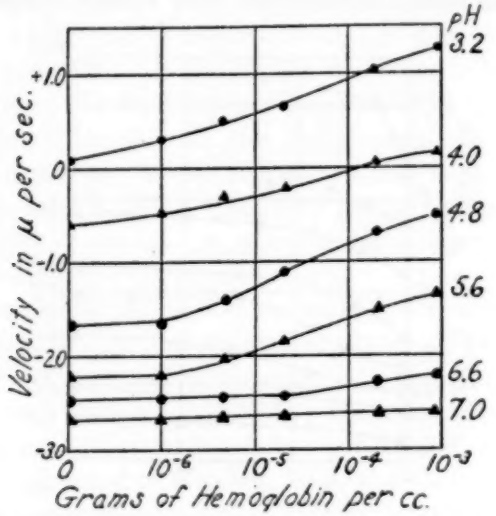

FIG. 2. The effect of hemoglobin on the velocity of human erythrocytes in 0.01 N phthalate and phosphate buffer mixtures.

were added to freshly prepared human erythrocyte suspensions in 0.01 N phosphate and phthalate buffers, and cataphoresed immediately.

The results given in Fig. 2 show that even in extreme dilution, hemoglobin has a great effect on the charge of erythrocytes. At pH 3.2, one part of hemoglobin in one million of solution, increases the positive charge threefold. At pH 4.0, this concentration of hemoglobin causes a small but definite diminution of the negative charge. At reactions nearer the isoelectric point of the hemoglobin (pH 6.8), increasing concentrations are necessary to bring about any alteration

in the mobility of the erythrocytes. At pH 7.0, no hemoglobin effects were observed at all.

On comparing Figs. 1 and 2, it will be seen that hemolysis of some of the erythrocytes, which takes place in the more acid solutions, fully explains the alteration in charge which the intact cells undergo. An amount of hemolysis so slight that the eye cannot detect it may very greatly alter the charge of the cells.

Whether acid-treated erythrocytes, freed from traces of hemoglobin, behave differently from fresh erythrocytes could not be demonstrated clearly. Experimental results obtained were conflicting; this

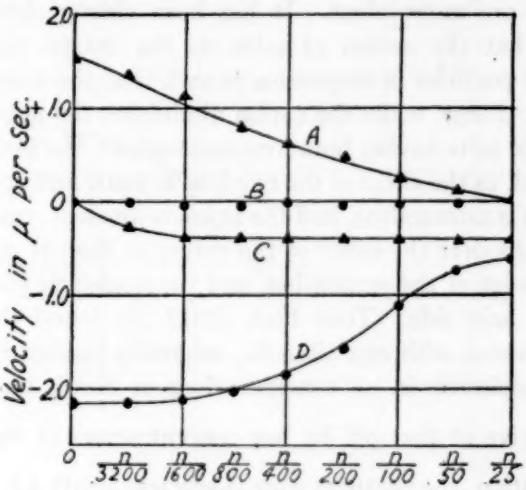

FIG. 3. The velocity of rabbit erythrocytes in salt solutions of different concentrations. *A*, potassium hydrogen phthalate at pH 3.6. *B*, potassium lactate at pH 4.7. *C*, potassium phthalate at pH 4.7. *D*, potassium phosphate at pH 6.6.

was probably due to the fact that acid treatment increases the fragility of the cells, and any manipulation hemolyzes some of them: the hemoglobin effect then obscures everything else.

As was stated before, the isoelectric points of sheep, human, and rabbit erythrocytes were found to lie at pH 3.2, 3.5, and 4.0, respectively, in the 0.01 N phthalate buffers. When it is recalled that Coulter (1920–21) found sheep erythrocytes in salt-free suspensions to be isoelectric at pH 4.65, an explanation of these observations becomes necessary.

Coulter's work was repeated using, however, the microcataphoresis apparatus instead of the apparatus used by him. It was found that human and rabbit, as well as sheep erythrocytes were isoelectric at about pH 4.7, both in the absence of salt and in various concentrations of potassium lactate. Yet in phthalate buffer at this pH, the erythrocytes have a considerable negative charge, which is manifest even in a salt concentration of $\frac{N}{3,200}$ (Fig. 3).

The reason for this difference lies in the fact that the phthalate buffer at this pH contains a large proportion of divalent anions while the cations are monovalent. It has been clearly shown by Loeb (1922–23) that the action of salts on the cataphoretic potential difference of particles in suspension is such that the anion diminishes the positive charge, while the cation diminishes the negative charge; in the case of salts having both ions monovalent, the isoelectric point is not shifted, as the effect of the two ions is equal and opposite. But if the cation is monovalent, and the anion is divalent, the effect of the anion prevails over the effect of the cation at the pH of the original isoelectric point of the suspension, and the isoelectric point is shifted toward the acid side. Thus Loeb (1922–23) found that collodion particles covered with egg albumin, originally isoelectric at pH 4.5, remained isoelectric in all concentrations of NaCl, but were made electronegative at that pH by low concentrations of Na_2SO_4; in $\frac{M}{128}$ Na_2SO_4 solution, the particles were isoelectric at pH 4.0, while a concentration of $\frac{M}{16}$ made them isoelectric at pH 3.0. Fig. 3 shows that the same principles apply in the case of erythrocytes. At pH 4.7, the original isoelectric point of the erythrocytes, potassium lactate in all concentrations tried, left the potential difference unaltered, while the potassium phthalate, even in low concentrations, conferred on the cells a negative charge. At pH 3.2 (Fig. 3, *A*), the anions of the phthalate solution are monovalent; at this pH, the monovalent anion is more effective than the monovalent cation, and the result is an increasing diminution of the positive charge with increasing salt concentration. This is analogous to the effect of sodium chloride on the charge of albumin-coated collodion particles on the acid side of

their original isoelectric point (Loeb, 1922–23). At pH 6.6 (Fig. 3, *D*), is shown the effect of potassium phosphate; the cation is shown to be the effective ion.

Fig. 4 again shows the effect of salt ions on the charge of erythrocytes. The salts all have the same equivalent concentration, 0.01 N. On the acid side of the isoelectric point, monovalent and divalent cations have the same effect, while the divalent anion greatly diminishes or even reverses the positive charge. At pH 4.7 to 5.1, the effect of the divalent anion is still seen; at more alkaline reactions it

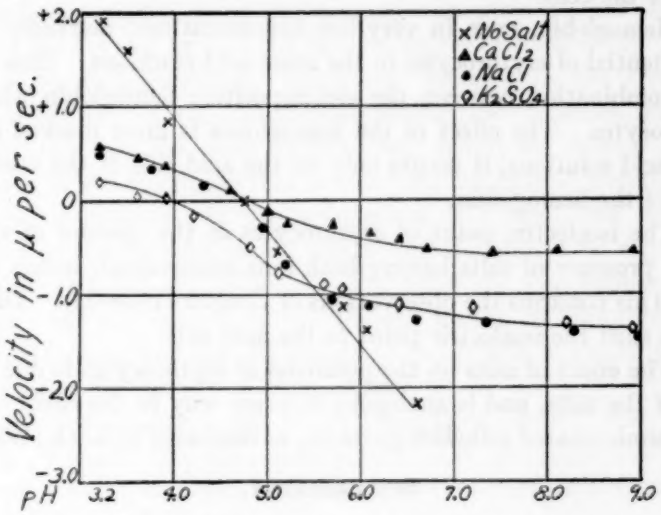

FIG. 4. The velocity of rabbit erythrocytes in 0.01 N $CaCl_2$, NaCl, and K_2SO_4 solutions, and in the absence of salt.

disappears, and equivalent concentrations of NaCl and K_2SO_4 are equally effective in depressing the negative charge. That this depressing effect of salts on the alkaline side of the isoelectric point is due to the cation is shown by the increased effect of the divalent calcium ion. It will be noted, however, that $CaCl_2$ in this concentration does not shift the isoelectric point to the alkaline side to any marked degree; it is interesting to note that Loeb (1922–23) also found no significant difference between the action of calcium and sodium ions on albumin-coated collodion particles at their isoelectric point.

SUMMARY.

1. Human and sheep erythrocytes, when placed in 0.01 N buffer solutions at reactions more acid than pH 5.2, undergo a progressive change in potential, becoming less electronegative or more electropositive. This change usually occurs within 2 hours at ordinary room temperatures. It did not occur when rabbit erythrocytes were used.

2. This change is due primarily to the liberation of hemoglobin from some of the cells.

3. Hemoglobin, even in very low concentrations, markedly alters the potential of erythrocytes in the more acid reactions. This is due to a combination between the electropositive hemoglobin and the erythrocytes. The effect of the hemoglobin is most marked in the more acid solutions; it occurs only on the acid side of the isoelectric point of the hemoglobin.

4. The isoelectric point of erythrocytes in the absence of salt, or in the presence of salts having both ions monovalent, occurs at pH 4.7. This confirms the observations of Coulter (1920–21). Divalent anions shift the isoelectric point to the acid side.

5. The effect of salts on the potential of erythrocytes is due to the ions of the salts, and is analogous in every way to the effect of salts on albumin-coated collodion particles, as discussed by Loeb (1922–23).

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THE GROWTH OF THE THYROID AND POSTBRANCHIAL BODY OF THE SALAMANDER, AMBYSTOMA OPACUM.

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(Received for publication, March 23, 1924.)

It seemed probable, from previous experimentation, that in salamanders the thyroid attains the highest degree of its activity during the time of metamorphosis. Since the decrease in the size and weight of the animal, the considerable destruction of tissues, and the exophthalmus are the most outstanding symptoms during metamorphosis, it was suggested that at this time the thyroid functions in an excessive manner. If this assumption is correct, the unusual condition of the thyroid during metamorphosis should be expressed in the growth curve of this organ. The following observations seem to prove that the growth of the thyroid during metamorphosis is characteristic and does not correspond to the growth of the animal as a whole, or to the growth of other organs.

The relative weights of the thyroid and postbranchial body were found by reconstructing the organs (exclusive of the connective tissue and blood vessels) from wax, after the method of Born, at a magnification of one hundred times.

The weights of both organs, relative to the size of the animal, were expressed by the quotients $\frac{\sqrt[3]{\text{weight of organ}}}{\text{body length}}$ and $\frac{\sqrt[3]{\text{weight of organ}}}{\text{total length of animal}}$. The first quotient is the better value, as the body length is more reliable than the total length. It was found that the quotients, when plotted against the age of the animals, give a curve closely approximating a curve obtained from the quotient $\frac{\text{weight of organ}}{\text{weight of animal}}$. The first curves, however, are better than the curve plotted from the weight of the animal, as the two former values are more significant than the weight.

In Fig. 1 the weights of the organs (wax models), the quotient $\frac{\sqrt[3]{\text{organ weight}}}{\text{body length}}$ for both organs, and the body length of the animals are plotted against the age. The material is composed of three series of *Ambystoma opacum*, which hatched at slightly different times, but are the offspring of the same female, and were kept under the same conditions; the older animals (A_4, W_2, $CXXXVI_2$, XIV_4, Stock 2, and W_{ca5}) are normal animals, but from different females and seasons. The animals represent stages from immediately after hatching up to $4\frac{1}{2}$ years. The beginning of metamorphosis was taken as coincident with the first skin-shedding. As it was found that the time elapsed since the first skin-shedding was of far greater influence upon the size of the thyroid than the age, the former value was substituted for the latter from the time of skin-shedding on.

The absolute weight of the thyroid gives a curve essentially similar to the type of curves well known for the growth of whole organisms, except that there is a rather considerable amount of variation at the time of the first skin-shedding. It is, however, noticeable that the most rapid rise takes place just at the time of skin-shedding (the last larval animal, of an age of 123 days, was just in the process of skin-shedding when killed). Both these conditions, the variation as well as the sudden rise, indicate that at this time an abrupt change must take place in the thyroid.

The same fact seems to be expressed even more clearly if the quotient $\frac{\sqrt[3]{\text{thyroid weight}}}{\text{body length}}$ is examined. Again it is noted that an abrupt rise of the curve takes place at the time of the first skin-shedding, which ends 30 days after the first skin-shedding.

The rise of both the absolute and the relative weight of the thyroid becomes the more noteworthy as the curve of the growth of the body shows a drop. The growth of the thyroid does not follow the cessation of growth and diminution of size of the whole animal which occurs during metamorphosis. This fact is well expressed in Fig. 2. Data from animals kept under the most varied conditions, hatched from eggs of different females, and at different seasons (from 1916 to 1922) have been used in this chart. Some of the animals have been very abnormal, due to the experimental conditions employed. Only those

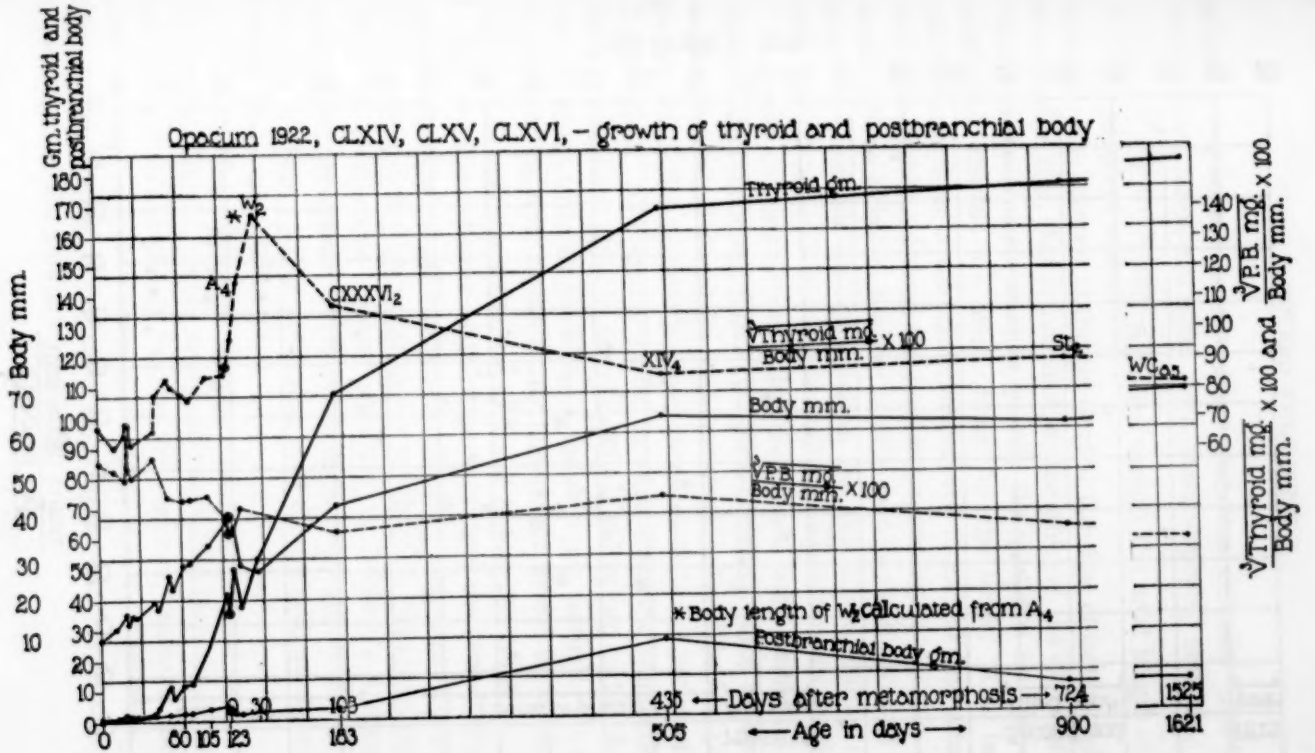
Opacum 1922, CLXIV, CLXV, CLXVI, – growth of thyroid and postbranchial body
Gm. thyroid and postbranchial body
Body mm.
Thyroid gm.
$\sqrt{\text{Thyroid mg.}}$ / Body mm. x 100
Body mm.
$\sqrt{\text{V.P.B. mg.}}$ / Body mm. x 100
* Body length of w_2 calculated from A_4
Postbranchial body gm.
A_4
w_2
CXXXVI$_2$
XIV$_4$
St$_2$
WC$_{92}$
$\sqrt{\text{V.P.B. mg.}}$ / Body mm. x 100 and $\sqrt{\text{Thyroid mg.}}$ / Body mm. x 100
Days after metamorphosis
Age in days
0
60
105
123
183
435
505
724
900
1525
1621

FIG. 1.

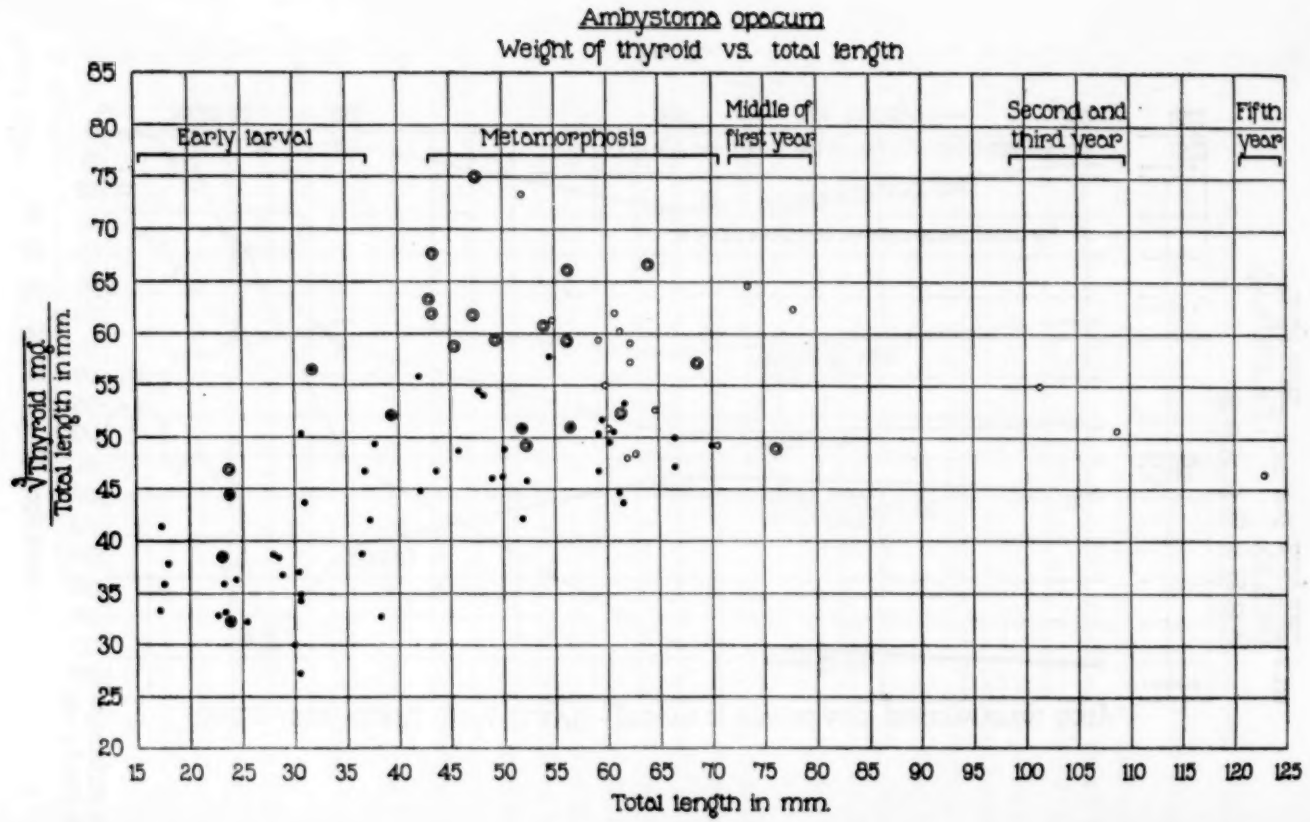
Ambystoma opacum
Weight of thyroid vs. total length
Early larval
Metamorphosis
Middle of first year
Second and third year
Fifth year
$\sqrt[3]{\frac{\text{Thyroid mg.}}{\text{Total length in mm.}}}$
Total length in mm.
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125

FIG. 2.

without a ring around the central disc are normal animals. The black discs indicate larvæ, the white discs, metamorphosed animals. The quotient $\frac{\sqrt[3]{\text{thyroid weight}}}{\text{length of animal}}$ is plotted against the total length of the animals. The increase of the quotient at the time of metamorphosis is so considerable, that it overshadows the effect of the differences in the experimental conditions; in general, the quotients for the metamorphosed animals are above those for the larvæ. The largest quotients are those of animals killed between 13 and 30 days after the first skin-shedding (along the line corresponding to 45 mm. total length); those killed on the day of skin-shedding and up to 13 days after have smaller quotients (along the line corresponding to 60 mm. total length). Later on the quotients again become smaller.

It should also be mentioned that the thyroid body quotient, before the first skin-shedding, shows two periods during which it seems to be constant, but different for both periods (Fig. 1). The first period, during which the quotient is lower, corresponds to the period of limb development. The second period extends from the completion of limb development close to the first skin-shedding (up to 123 days of age, at which time the shedding of the skin just started). It is not possible, at present, to say if any importance should be ascribed to this fact, as only one series was available to test this point.

The peculiar behavior of the thyroid during the period of metamorphosis becomes still further elucidated by comparing the growth of the postbranchial body with that of the thyroid. The postbranchial body of *Ambystoma opacum*, contrary to Baldwin's findings[1] in *Ambystoma maculatum*, was found to persist throughout life up to $4\frac{1}{2}$ years. Neither atrophy nor fusion with the thyroid, as observed in higher vertebrates, takes place.

As compared with the thyroid, the postbranchial body grows very little (Fig. 1). It not only lacks the sudden increase at the time of the first skin-shedding, but, on the contrary, shares in the drop exhibited by the growth curve of the whole animal at that time. Like the animal's body, it begins to grow again some time after skin-shedding, but the growth is very slow.

[1] Baldwin, F. M., *J. Morphol.*, 1917–18, xxx, 605.

In comparing the curve of the postbranchial body quotient with the corresponding curve of the thyroid, it is noted that it moves in exactly the opposite direction. It, too, shows two periods before metamorphosis corresponding exactly to the periods of the thyroid quotient; but for the postbranchial body the quotient is lower during the second period. On the whole it seems that the larger the relative size of the thyroid, the smaller the relative size of the postbranchial body. This point, however, needs further tests before any significance can be attached to it.

The growth of both the body of the animal and of the postbranchial body are interrupted at the time of metamorphosis. The thyroid, however, does not share in the cessation of growth; consequently, the thyroid body quotient shows a sudden and considerable rise beginning at the time of the first skin-shedding.

The writer feels indebted to Miss Frances E. McGowan for valuable assistance rendered in the course of his work.

THE AGGLUTINATION OF RED BLOOD CELLS.

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(Received for publication, March 14, 1924.)

The agglutination of a number of suspensions has been found to be closely related to the potential difference between the particle and the surrounding liquid as measured by the migration in an electric field.[1] If the potential is higher than a certain value—the critical potential—the particles remain separate, if it is lower than this value, they adhere or agglutinate. It follows that agglutination may be caused in two ways, (1) by decreasing the actual potential, or (2) by increasing the critical potential. In general, electrolytes do not affect the value of the critical potential so that in most cases agglutination occurs whenever sufficient electrolyte has been added to depress the potential below a certain definite value. This has been found in many cases to be about 14 millivolts. The value is usually independent of the nature of the electrolyte and even of the nature of the particle. It was found in the study of agglutination of *Bacillus typhosus*,[2] however, that in this case high concentrations of electrolyte decrease the critical potential as well as the actual potential, so that no agglutination occurred in strong NaCl although the potential was practically zero. In confirmation of this, it was found that the force with which two smears of the organism stuck together was also decreased by concentrated salt. Bacteria which had been sensitized with immune serum do not show this peculiarity and agglutination occurs whenever the potential is reduced below about 14 millivolts.[3]

The effect of the antiserum is, therefore, primarily on the critical potential whereas the effect of electrolytes is primarily on the poten-

[1] A general summary of the agglutination of colloidal particles can be found in Bogue, R. H., Theory of colloidal behavior, New York and London, 1924, chapter on the flocculation and stability of colloidal suspensions.

[2] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

[3] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 655.

tial itself. The agglutination of red cells is essentially similar to that of bacteria and it has already been shown by Mines[4] and especially Coulter[5] that the acid agglutination point coincides with a very low potential. The present paper contains the result of experiments in which the effect of electrolytes, serum, ricin, colloidal stannic hydroxide, and paraffin oil on the potential and agglutination of red blood cells has been studied. It has been found that agglutination of unsensitized cells occurs if the potential is depressed below about 4 millivolts whereas cells which have been previously treated with immune serum, ricin, colloidal stannic hydroxide, or paraffin oil agglutinate when the potential is below about 12 millivolts.

Experimental Procedure.—The blood was collected in citrate, centrifuged, and the cells washed with 4 per cent dextrose containing M/10,000 trisodium citrate, in order to prevent agglutination by the CO_2. The suspension was then diluted to twenty times the original volume of the blood and 1 cc. of this added to 50 cc. of the various salt solutions. The potential was measured as described in a previous publication by noting the rate of migration in an electric field. The potential was calculated by means of the formula P.D. in millivolts $= \frac{13 \times \mu \text{ per second}}{\text{volt per centimeter}}$. There is considerable uncertainty as to the correctness of this formula and the results could be equally well expressed as rate of migration instead of potential difference. The tubes were allowed to stand about 18 hours at 10°C. and the readings made after this time. C was recorded when the suspension had settled, leaving a perfectly clear supernatant liquid. In some cases the suspension was filtered through a coarse grade of filter paper. Those tubes read as C gave a clear filtrate containing no cells.

Agglutination by Electrolytes.

Cells suspended in sugar alone frequently showed agglutination due to CO_2 adsorption and subsequent reduction of the pH to the acid agglutination range.[5] In order to avoid this difficulty, a very low concentration of trisodium citrate[6] (10^{-4} M) was added to the

[4] Mines, G. R., *Kolloidchem. Beihefte*, 1911–12, iii, 191.
[5] Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.
[6] Atkin, E. E., *Z. Immunitätsforsch., Orig.*, 1908–09, i, 387.

suspension. This salt confers a negative charge on the cells and renders the suspension stable. The variations in the potential of the control tubes are due to variations in the amount of citrate present.

The effect of electrolytes is shown in Table I. It is evident that whenever the potential is less than about 6 millivolts the cells agglutinate, except in the case of $MgCl_2$ and $CaCl_2$. In these two salts no agglutination occurs even though there is no potential. The same peculiarity was found in regard to bacteria and is due to a decrease in the critical potential by these salts. It could be shown in the case of bacteria that this decrease was paralleled by a decrease in the force required to tear apart two films of the organisms.[2] In the presence of concentrated Ca or Mg salts, therefore, there is no "cohesive force" between the particles and so no potential difference is required to keep them apart. That this is not due to an error in the P.D. measurement may be shown by adding increasing amounts of HCl to a suspension in 0.10 M $MgCl_2$. Under these conditions the potential will vary from very slightly negative to slightly positive and must therefore be zero in some intermediate tube even though there is a large error in the measurement. No agglutination occurs, however.

As usual the concentration required to agglutinate is less the higher the valence of the oppositely charged ion (cation), and salts of heavy metals are more efficient than alkaline earth salts. This agglutinating effect of salts had been noted by Eisner and Friedemann[7] and by Schürmann and Baumgärtel.[8] The present experiment shows that it is due to the reduction of the potential below the critical value. Excess of polyvalent cations or hydrogen ions confers a positive charge and the suspension may again become stable.

Agglutination by Immune Serum.

Table II shows the relation between the potential and agglutination by immune serum. In NaCl under the ordinary conditions of experiment the serum causes agglutination both by decreasing the

[7] Eisner, G., and Friedemann, U., *Z. Immunitätsforsch., Orig.*, 1914, xxi, 520. *Cf.* also Landsteiner and Jagić.[13]

[8] Schürmann, W., and Baumgärtel, T., *Z. Immunitätsforsch., Orig.*, 1921, xxxi, 151.

TABLE I.

Effect of Various Electrolytes on Potential and Agglutination of Sheep Cells in Isotonic Sugar.

1/1,000 red cells in isotonic solution containing 10^{-4} M Na_3 citrate.

Electrolyte.	NaCl		NH_4Cl		$MgCl_2$		$CaCl_2$		$CuSO_4$		Pb acetate.		HCl (0.001 citrate.)		$LaCl_3$		$Th(NO_3)_4$	
Concentration.	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.
M	mv.		mv.		mv.		mv.		mv.		mv.		mv.		mv.		mv.	
—	17.0	—	−15.0	—	17.8	—	−12.2	—	−11.0	—	−16.3	—	−44.0	—	−16.0		−13.8	—
5×10^{-7}													−32.0	—			−14.0	—
3×10^{-6}																	−6.0	+
5×10^{-6}									8.0	—							0	C
10^{-5}											−17.0	—	−39.0	—			+4.7	C
3×10^{-5}									−4.2	+			−22.0	+	−12.6	—		
6×10^{-5}													−6.0	++			+12.0	—
10^{-4}									0	C	−8.0	—	0	C	−7.1	+		
3×10^{-4}									+3.0	C			0	C				
10^{-3}					−13.8	—	−12.2	+	+3.5	C	−1.0	C	+12.0	(Hemolysis.)	0	C		
3×10^{-3}			−13.0	—														
6×10^{-3}					−8.7	—	−9.2	+							+2.0	+++		
10^{-2}	15.0	—	−8.0	—	−2.5	—	8.4	+	+4.0	C	0	C						
3×10^{-2}	15.0	—			0	—	3.8	+							+3.0	+++		
6×10^{-2}	12.0	—	−8.4	—	0	—	2.5	+										
10^{-1}	11.0	—			0	—					0	C						

potential and increasing the critical potential. Normal serum has no effect on either value. In $MgCl_2$ there is no change in the actual potential and yet agglutination occurs. This experiment shows that the effect of the immune serum is primarily to raise the critical potential. This effect is shown more clearly if the cells are first sensitized with antiserum in sugar and then treated with increasing salt concentrations. A large excess of inactivated antiserum, equivalent to ten agglutinating doses, was added to a suspension of sheep cells in citrated sugar. The suspension was centrifuged and the cells washed with sugar. 1 cc. of this suspension was then added to the 50 cc. of the salt solutions. The results are shown in Table III.

TABLE II.

Effect of Immune Serum on Agglutination and Potential of Sheep Cells Suspended in NaCl or $MgCl_2$.

Concentration of rabbit anti-sheep serum. (Cc. serum in 50 cc. of suspension.)	Sheep cells in 0.85 per cent NaCl.		Sheep cells in M/15 $MgCl_2$.	
	P.D.	Agglutination.	P.D.	Agglutination.
M	mv.		mv.	
–	−13.0	–	–	–
10^{-4}	−11.8	+	–	++
10^{-3}	−11.0	+	–	+++
10^{-2}	−6.7	+++	–	C
5×10^{-2}	−3.0	C	–	C
10^{-1}	−2.0	C	–	C
Normal rabbit serum.				
10^{-1}	−12.7	–	–	–

Agglutination now occurs whenever the potential is less than about 12 millivolts instead of 6, as was the case with unsensitized cells, and the zone of agglutination is therefore broadened. This is the value that was found for sensitized *Bacillus typhosus*[8] and also for collodion particles and particles of denatured egg albumin.[9]

Agglutination by Ricin.

It was found by Stillmark[10] that rabbit cells are agglutinated by ricin and that the reaction was very similar to immune agglutination.

[9] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 479.

[10] Stillmark, H., *Arb. pharmacol. Inst. zu Dorpat*, 1889, iii, 59.

TABLE III.

Effect of Electrolytes on P.D. *and Agglutination of Sensitized Red Cells.*

Sheep cells washed in sugar + M/10,000 citrate. Three agglutinating doses of rabbit anti-sheep serum added. Centrifuged, washed, and resuspended in sugar + M/10,000 citrate.

Concentration, mols. per liter.	NaCl		NH_4Cl		$MgCl_2$		$CuSO_4$		HCl (0.0002 citrate.)		$Th(NO_3)_4$	
	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.
	mv.		mv.		mv.		mv.		mv.		mv.	
–	−23.0	–	14.0	–	−22.0	–	−14.0	–	−35.0	–	−16.0	–
3×10^{-6}							−12.0	+++				
10^{-5}											−12.0	+
3×10^{-5}							−8.0	C	−25.0	–	−10.0	C
6×10^{-5}									−17.0	+++	0	C
10^{-4}							−5.0	C	−6.0	C		
3×10^{-4}					−12.0	+++	–	C	+9.0	C	+29.0	–
6×10^{-4}									+25.0	+		
									(Hemolysis.)			
10^{-3}	−20.0	–	−13.0	–								
3×10^{-3}			−11.0	C	−6.0	C	–	C				
10^{-2}	−17.0	+++	−8.0	C	0	C						
3×10^{-2}	−11.3	C			0	C						
6×10^{-2}	−9.0	C	7.0	C	0	C						
10^{-1}	−7.0	C			0	C						
5×10^{-1}	−6.0	C			0	C						

Ricin does not combine with sheep cells and therefore does not agglutinate them. The "specific" reaction is again the combination rather than the effect. This combination is probably not connected with any electrical properties since ricin will agglutinate cells which have been given a positive charge by the addition of $Th(NO_3)_4$. The same experiment may be performed with antiserum.[11] The specific part of the reaction is evidently the combination since, as will be

TABLE IV.

Effect of Electrolytes on P.D. *and Agglutination of Rabbit Cells Sensitized with Ricin.*

Rabbit cells in dextrose containing 0.0001 M Na_3 citrate + > two agglutinating doses of ricin. Centrifuged, resuspended in citrate-sugar solution. Electrolyte added.

Concentration.	NaCl		HCl		$CuSO_4$	
	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.
M	mv.		mv.		mv.	
—	−26.0	0	−22.0	0	−22.0	—
4×10^{-5}			−18.0	—		
10^{-4}					−19.0	+
2×10^{-4}			11.7	+++	−15.0	++
4×10^{-4}			10.0	C	−14.0	C
10^{-3}			0	C	−6.5	C
2×10^{-3}	−17.0	++				
4×10^{-3}	−12.0	C				
10^{-2}	−9.0	C				
2×10^{-2}	−2.5	C				
10^{-1}	2.0	C				

seen below, the effect of antibody on the cells does not differ from that of several other substances. Table IV contains the results of experiments with rabbit cells sensitized with ricin. The critical potential is again about 12 millivolts as was the case with sensitized sheep cells.

[11] Similar experiments can be made with bacteria. *Cf.* De Kruif, P. H., and Northrop, J. H., *J. Gen. Physiol.*, 1922–23, v, 127.

Agglutination with Colloidal Stannic Acid.

Landsteiner and Jagić[12] showed that typical hemagglutination could be caused by colloidal solutions of various acids and metal hydroxides. Table V shows the effect of colloidal stannic hydroxide on the agglutination and P.D. of sheep cells. In NaCl complete agglutination occurs even in high dilution of stannic hydroxide without any effect on the potential. The critical potential is evidently raised. No agglutination occurs in sucrose because the potential is higher than that of the critical value even after the latter has been raised by the stannic

TABLE V.

Agglutination by Stannic Acid.

Stannic acid prepared by precipitation of $SnCl_4$ with NaOH, precipitate washed and resuspended by NH_4OH. Excess NH_4OH boiled out.

Dilution of stannic acid solution.	Sheep cells in 0.9 per cent NaCl.	
	P.D.	Agglutination.
	mv.	
–	−12	–
1/3,200	−12	–
1/1,600	−12	+
1/800	−12	C
1/400	−12	C
1/100	−12	C
	Cells in isotonic sucrose + citrate.	
–	−41	–
1/80	−40	–
1/10	−50	–

hydroxide. It has been found in a number of instances that colloidal particles assume the characteristics of other substances added to the solution. Bacteria in the presence of proteins, for instance, assume the isoelectric point of the protein,[3] as do collodion particles. A clear cut case was described by Loeb,[9] who found that collodion particles treated with gelatin were agglutinated by the same concentration of salt which was required to salt out solutions of gelatin. It might be expected, therefore, that the same concentration of salt which agglutinated cells sensitized with stannic hydroxide would also coagulate

[12] Landsteiner, K., and Jagić, N., *Wien. klin. Woch.*, 1904, xvii, 63.

the stannic hydroxide itself. That this is the case is shown in Table VI. This relation does not always hold, however, as it was found by Landsteiner that colloidal silicic acid sensitized cells to the action of

TABLE VI.

Effect of Electrolytes on P.D. *and Agglutination of Cells Sensitized with Colloidal Stannic Acid, and on Precipitation of Stannic Acid Solution.*

Concentration.	NaCl — red cells.		NaCl agglutinates stannic acid alone.
	P.D.	Aggl.	
M	mv.		
—	52.0	—	—
0.007	30.0	—	—
0.015	24.0	+	—
0.03	20.0	+	—
0.06	11.7	C	C
0.12	8.0	C	C
0.25	<8.0	C	C
0.50	<8.0	C	C

TABLE VII.

Effect of Electrolytes on P.D. *and Agglutination of Sheep Cells Sensitized with Stannic Acid.*

Electrolyte concentration.	NaCl		HCl		$MgCl_2$	
	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl
M	mv.		mv.		mv.	
—	−52	—	−35	—	−38.0	—
10^{-4}			−18	—		
2×10^{-4}			−12	C		
4×10^{-4}			−8	C		
2×10^{-3}					−14.0	++
4×10^{-3}					−10.6	C
10^{-2}	−30	—			−9.0	C
2×10^{-1}	−24	—			−8.0	C
4×10^{-1}	−20	++				
10^{-1}	−12	C			<−8.0	C
2×10^{-1}	−6	C				
5×10^{-1}	<−6	C				

electrolytes without being itself precipitated. The effect of electrolytes on the potential and agglutination of cells sensitized with stannic hydroxide is shown in Table VII.

Agglutination of Cells Sensitized with Paraffin Oil.

It was shown by Powis[13] that oil drops have a very high critical potential since they agglutinate when the potential is less than about 30 millivolts. It would be expected, therefore, that if the cells could be coated with a film of oil that they would become also very unstable. This is partially true, as is shown in Table VIII. The critical potential is raised to about 12 millivolts as was the case with antiserum, ricin, or stannic hydroxide.

TABLE VIII.

Effect of Electrolytes on P.D. *and Agglutination of Sheep Cells Sensitized with Paraffin Oil.*

Suspension of sheep cells in citrated sugar shaken with a large excess of heavy paraffin oil (Nujol). The excess oil removed, suspension washed twice in sugar, and noted electrolytes added to the cell suspension.

Concentration of electrolyte.	NaCl		$MgCl_2$		HCl	
	P.D.	Aggl.	P.D.	Aggl.	P.D.	Aggl.
M	mv.		mv.		mv.	
—	−38.0	−	−49.0	−	−49.0	−
10^{-4}					−17.0	−
10^{-3}					+12.0	C
2×10^{-3}			−15.0	−		
4×10^{-3}			−14.0	+		
10^{-2}			−11.0	C		
2×10^{-2}	−22.0	−	−6.3	C		
4×10^{-2}	17.0	++	<5.0	C		
10^{-1}	13.0	C	<5.0	C		
2×10^{-1}	11.0	C	<5.0	C		

The effect of all these substances is therefore similar in that they increase the critical potential of the cells. This effect is evidently closely analogous to the more general one of "protective colloids." In this latter case the critical potential is decreased instead of increased and the suspension thereby rendered less sensitive to electrolytes instead of more sensitive.

[13] Powis, F., *Z. physik. Chem.*, 1914–15, lxxxix, 179, 186.

SUMMARY.

1. Unsensitized sheep cells suspended in sugar solutions are agglutinated by electrolytes whenever the potential is depressed to 6 millivolts or less, except in the case of $MgCl_2$ or $CaCl_2$.

2. With these salts no agglutination occurs although there is practically no potential. The presence of these salts prevents acid agglutination. This is presumably due to a decrease in the "cohesion" between the cells.

3. Cells which have been sensitized with specific antibody, ricin, colloidal stannic hydroxide, or paraffin oil, are agglutinated whenever the potential is decreased below about 12 millivolts.

4. The agglutination by electrolytes is therefore primarily due to a decrease in the potential whereas agglutination by immune serum, ricin, etc., is due primarily to an increase in the critical potential.

THE LOCUS OF THE ACTION OF VERATRIN.

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Maxwell (1) has shown that some nerve stimulants, such as the calcium precipitants, stimulate medullated nerve fibers alone, and he has termed these substances nerve excitants of the first class. Others, such as creatine, strychnine and picrotoxin stimulate only nerve cells and these form the second class. Tetraethyl ammonium chloride, since it stimulates both medullated nerve and nerve cells must be included in both classes. Veratrin for the reason that it directly stimulates some of the ganglia in the medulla of vertebrates must be classed as a neurophil alkaloid of the second group (2). Furthermore, in all treatises on the subject, so far as I have been able to discover, it has been assumed that veratrin also acts in a characteristic way directly on the striated muscle fibers of the vertebrates (3). It remains to be determined whether veratrin stimulates medullated nerve fibers. If this should be the case, the question is at once raised as to whether the supposed action of veratrin on muscle may not be referable to its action on nerve fibers.

In order to throw some light on the problem of the locus of action of veratrin in different forms, experiments were made with specimens of *Mnemiopsis leidyi*, *Lumbricus terrestris*, and *Musca domestica* from the invertebrate group, and frogs (*Rana pipiens*) from the vertebrate group.

All veratrin solutions were made from a sample of the amorphous powder obtained from the Mallinckrodt Chemical Company. The concentration used unless otherwise stated was 1 part by weight of veratrin to 1,345 parts of sea water or Ringer's solution as the case may be.

Mnemiopsis leidyi.

If specimens of *Mnemiopsis leidyi* (4) be immersed in a solution of veratrin made up in sea water the swimming plates immediately stop in an upright position. But, if the animal be cut into small pieces, in 2 minutes the isolated single plates and groups of plates begin beating, and continue to beat for at least half an hour in the solution of veratrin.

In the case of intact specimens there is only slight, irregular movement at the extreme tips of the plates after 15 or 20 minutes immersion in veratrin. This slight movement at the tips increases with time, although the effective stroke is at first deficient, and at the end of an hour is abnormally fast and vigorous. If during this stage, there is momentary cessation of beat, the plates do not lie prone as in the normal animal, but stop in the upright position. It can also be seen that slight mechanical stimulation at this time causes, not cessation of beat, but excitation and acceleration of beat. This abnormally vigorous type of motion of the swimming plates is maintained for a period of 6 or 7 hours, even appearing in isolated plates after disintegration of the animal.

It was also observed that the normal action of the food cilia was not influenced by immersion of the animal in veratrin. This suggests that the control mechanisms in the case of food cilia and swimming plates are not the same.

Since the first brief effects of veratrin, namely inhibition of beat, can be removed by isolation of plates, and since in the secondary stage of veratrin poisoning, mechanical stimulation causes not inhibition but excitation, it seems probable that the locus of veratrin action in *Mnemiopsis leidyi* is on the nervous mechanism.

Lumbricus terrestris.

It has been shown that the earthworm is a form favorable for the demonstration of the action of neurophil alkaloids, since the exposed ventral nerve cord is stimulated by camphor, strychnine, atropine, and picrotoxin (5).

If an intact worm be immersed in a watery solution of veratrin the result is a prolonged series of spasmodic contractions, involving both

circular and longitudinal muscles. Similar results are obtained in decapitated specimens, or in smaller pieces. Mechanical stimuli at this time do not elicit peristaltic movements, as in a normal worm, but cause spasmodic contractions of the musculature.

The action of veratrin on the ventral nerve cord was tested as follows: A decapitated animal was pinned down by the anterior and posterior ends and the intestine and ventral nerve cord removed. This left a sheet of muscle tissue, which was clamped at the posterior end and fastened by the anterior end to a counterbalanced writing lever, the whole apparatus being arranged for making a kymographic record. If this preparation of worm is now immersed, first in Ringer's solution then in a solution of veratrin, the result is that the writing lever does not leave the base line in either case. But, if the same procedure be repeated using a piece of worm containing the nerve cord, the response is of an entirely different character. Immersion in Ringer's solution results in normal peristalsis, while immersion in the solution of veratrin produces abnormally vigorous and prolonged contractions of the longitudinal muscles (Fig. 1).

In order, finally, to determine whether veratrin acts on the ventral nerve cord alone, experiments were made in which a worm was decapitated and the anterior portion of the ventral cord was exposed for a distance of about 3 cm. A piece of the cord about one-half this length was dissected free from the surrounding tissue, the latter being entirely removed leaving $1\frac{1}{2}$ cm. of the nerve cord free. The specimen was arranged in the usual way for making kymographic records. If now the free portion of nerve cord be immersed in Ringer's solution, normal peristalsis follows; but, if Ringer's solution be replaced by a solution of veratrin the result is a very great and immediate increase in the strength of circular and longitudinal muscular contractions (Fig. 2).

If the free end of the nerve cord of an earthworm preparation be treated with a solution of veratrin, characteristic reactions of both circular and longitudinal muscles follow. On the other hand, identical treatment of a muscle preparation from which the nerve cord has been removed has no result in stimulation of the muscles. It must therefore be concluded that veratrin does not act on the musculature of

the worm nor on nerve endings in the muscle, but upon the nerve cord alone. Hence veratrin, in the case of *Lumbricus* acts as a neurophil alkaloid of the second group.

Musca domestica.

Several house-flies kept under ventilated glass jars were given a syrupy solution of veratrin. This they drank readily. Within 5 minutes the first effects were apparent. The symptoms developed in three distinct stages.

1. Extreme elevation of the anterior portion of the body, the action so pronounced that it is suggestive of opisthotonos.

2. Loss of control of legs, which are spread apart. Repeated spasmodic attempts to fly.

3. Complete loss of equilibrium, usually ending with the fly lying ventral side upward, with legs flexed close to body. At this time if an individual be stimulated mechanically the result is shown in convulsive spasms and inability to make progressive movements. If now, these individuals be decapitated, the legs relax and extend so that the animal regains its equilibrium. In some cases there are more or less successful attempts to fly. In fact, from successful attempts of this kind specimens were lost to the experiment. If the third stage is allowed to progress too long, the animal dies and consequently decapitation is without effect.

Since the effects of poisoning by veratrin, namely rigid flexion of the legs and loss of equilibrium, can be removed by decapitation, the necessary conclusion is that in *Musca domestica* veratrin acts on the brain alone, and therefore as a neurophil alkaloid has a specific action on the neurones of the head ganglia. For this reason veratrin must be included in the second group of nerve excitants.

Nerve Fibers of Rana pipiens.

It has been shown that nerve fibers of the frog's nerve-muscle preparation can be stimulated chemically. For example, a solution of sodium citrate applied to the sciatic nerve results in irregular contractions of the gastrocnemius muscle. Similarly, if the sciatic nerve is immersed for 20 minutes in a solution of veratrin made up in Ringer's

solution, more or less rhythmical contractions of the gastrocnemius result. This can be demonstrated in the following way. The usual nerve-muscle preparation of the frog is made, and the preparation set up in such fashion that the nerve lies in a glass nerve holder containing the solution of veratrin. Precautions are taken to see that the nerve is free from tension, and that it does not become dry in the space between the nerve holder and the muscle. A vaseline ring is placed around the nerve to make sure that none of the solution of veratrin reaches the muscle by capillary action. After a latent period of 15 to 25 minutes spontaneous muscle twitchings begin in the gastrocnemius (Fig. 3).

It can be proven that veratrin does not spread *through* the nerve fibers to the muscle. For if during the muscle twitchings shown in Fig. 3, the nerve be clipped between the point of immersion in the veratrin solution and the point of attachment to the preparation, the writing lever immediately falls to the base line and remains at rest (Fig. 4). This proves that the action of veratrin in this case is on the medullated nerve fiber and not on muscle. Veratrin, therefore, is a neurophil alkaloid of the first class. Since it also belongs to the second class, veratrin is in the same category as tetraethyl ammonium chloride.

In further experiments it was found that a typical veratrin contracture results if the muscle be directly stimulated with single induction shocks ("break shocks," with coil set at 8 cm.) after the sciatic nerve is immersed in veratrin solution for a few moments, but before the spontaneous muscle contractions begin. If however, the sciatic be sectioned and electrical stimuli again applied, the contracture disappears and the period of relaxation becomes normal (Fig. 5). This proves that the characteristic veratrin curve of the frog's gastrocnemius can be reproduced without the muscle itself ever having been subjected to the action of veratrin, if only the end of the sciatic nerve be veratrinized.

It has been observed by previous investigators, that with gastrocnemius muscles of frogs veratrinized by injection into the dorsal lymph sac the descending limb of the curve of muscle contraction often showed distinct and somewhat rhythmical waves (6). Such rhythmical waves can also be observed in nerve-muscle preparations, when

electrically stimulated, if the sciatic nerve alone be immersed in a solution of veratrin (Fig. 6). From this it is clear that the characteristic waves in the relaxation curve of the muscle are caused by the action of veratrin on the nerve fibers alone. Presumably the waves in the relaxation curve of muscle, observed by former investigators, were caused by the same mechanism, namely the action of veratrin on the nerve fibers.

These results make it seem possible that the characteristic effects of veratrin heretofore ascribed to action of the drug on muscle cells may be due instead to the excitatory action of veratrin on the nerve fibers within the muscle.(7)[1] An answer can be given to the question by means of experiments on the sartorius muscle, since the upper part of this muscle contains nerve fibers while the lower part does not. With this purpose in view an entire sartorius muscle was dissected out and arranged in the usual way for making kymographic records.

If such a muscle preparation be bathed with a solution of veratrin the muscle always responds with a prolonged contraction as a result of veratrin stimulation (Fig. 7). Electrical stimulation applied after the contraction has ceased results in the characteristic veratrin muscle curve (Fig. 8). If a fresh sartorius be sectioned transversely through the middle, and the lower half which presumably consists of muscle cells without nerve fibers, be bathed in a solution of veratrin, there is no contraction as with the entire muscle, and electrical stimulation as a rule gives a normal contraction and relaxation (Fig. 9). The occasional exceptions to this rule may perhaps be accounted for on the ground that the region of the sartorius muscle which is supplied with nerves varies in different preparations, and that it is impossible in every case to section the muscle exactly on a line between the part containing nerve fibers and that which does not.

On the other hand if the upper half of the muscle which is supplied with nerve fibers is bathed in a solution of veratrin the result is always a prolonged contraction (Fig. 10), and subsequent electrical stimulation elicits a contraction with a delayed relaxation period (Fig. 11).

[1] Wyman (7) has found that the action of veratrin in causing an expansion of chromatophores is probably caused by excitation of the nerves supplying the chromatophores.

The facts show then that while the entire sartorius and the upper half of the muscle respond in characteristic fashion to veratrin poisoning, the lower half of the muscle which contains no nerve fibers does not contract spontaneously when bathed in veratrin solution, nor does the lower half when stimulated electrically show a delayed relaxation in the muscle curve. For these reasons it must be concluded that veratrin produces its characteristic effects upon an entire muscle through its excitatory action on medullated nerve fibers of the muscle and that it is very questionable whether veratrin is capable of affecting functional striated muscle fibers.

SUMMARY.

1. In *Mnemiopsis* veratrin shows two stages of veratrin poisoning. First, inhibition of the beats of the plates which disappears on cutting them away either singly or in small groups. Second, after half an hour mechanical stimulation excites the beat of the plates in the intact veratrinized animal. It is concluded that veratrin acts on nervous tissue and not on the substance of the swimming plates.

2. In *Lumbricus*, veratrin acts on the ventral nerve cord alone, and not on the muscles and peripheral nerves.

3. In *Musca*, veratrin first causes opisthotonos, then spasms and extreme flexion of the legs. Decapitation causes these effects to disappear hence veratrin acts on the cerebral ganglia of the fly.

4. Veratrin applied to the sciatic nerve of the frog causes, after a latent period of 20 minutes, irregular contractions of the gastrocnemius which persist for an hour or more. Veratrin is thus a neurophil alkaloid of the first class as well as second and in this way resembles tetraethyl ammonium chloride.

5. If the end of a sciatic nerve is dipped into veratrin solution, then direct stimulation of the gastrocnemius muscle results in contraction with delayed relaxation, although the muscle itself is not subject to the action of veratrin.

6. By means of preparations of the sartorius muscle of the frog it is shown that veratrin acts not on the muscle cells directly but on the nerve fibers. Hence veratrin produces the characteristic muscle curve showing delayed relaxation by its action on the nervous elements.

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EXPLANATION OF FIGURES.

FIG. 1. Curve of contraction of a preparation of earthworm containing nerve cord, showing effect of immersion in veratrin solution in greatly increased contraction of the longitudinal musculature. X indicates point at which veratrin was applied. Tracing made from right to left.

FIG. 2. Earthworm preparation with nerve cord freed at one end. When this part of the cord is immersed in veratrin solution (shown at X) the strength of peristalsis increases and tone is heightened.

FIG. 3. Tracing made by frog's gastrocnemius muscle after its sciatic nerve had been immersed in a veratrin solution for 20 minutes.

FIG. 4. Shows effect of sectioning (at X) the sciatic nerve of the preparation described in Fig. 3. The muscle at once relaxes.

FIG. 5. *a*, normal contraction curve of frog's gastrocnemius resulting from direct electrical stimulation of the muscle. *b*, same with the sciatic nerve immersed in veratrin showing delayed relaxation characteristic of veratrin poisoning. *c*, contractions of same after section of the sciatic.

FIG. 6. Showing periodic waves in the relaxation phase of muscle with sciatic nerve immersed in veratrin.

FIG. 7. Contraction curve of the frog's sartorius muscle obtained as a result of immersion in a solution of veratrin.

FIG. 8. *a*, normal contraction of the sartorius muscle, as a result of direct electrical stimulation. *b*, same after muscle has been immersed in veratrin.

FIG. 9. Contractions resulting from electrical stimulation of the veratrinized lower half of the sartorius which is not supplied with nerve fibers.

FIG. 10. Contraction obtained by immersion of the upper half of the sartorius in a solution of veratrin. This part of the sartorius contains nerve fibers.

FIG. 11. Electrical stimulation of the upper half of a sartorius which has been immersed in veratrin.

FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6.

FIG. 7.

a b

FIG. 8.

FIG. 9.

FIG. 10.

FIG. 11.